Upregulation of voltage-gated Na$^+$ channels by long-term activation of the ghrelin-growth hormone secretagogue receptor in clonal GC somatotropes

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1Laboratory of Neuroendocrinology, Institute of Physiology, Autonomous University of Puebla, Puebla, Mexico; 2Laboratory of Physiology, School of Veterinary Medicine and Zootechnology, University of Veracruz, Veracruz; and 3Department of Cell Biology, Center for Research and Advanced Studies of The National Polytechnic Institute, Mexico City, Mexico

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Domínguez B, Felix R, Monjaraz E. Upregulation of voltage-gated Na$^+$ channels by long-term activation of the ghrelin-growth hormone secretagogue receptor in clonal GC somatotropes. Am J Physiol Endocrinol Metab 296: E1148–E1156, 2009. First published February 17, 2009; doi:10.1152/ajpendo.90954.2008.—A central question in adenohypophyseal cell physiology concerns the role of transmembrane ionic fluxes in the initiation of the hormone secretion process. In the current report, we investigated the effects of the growth hormone (GH) secretagogues ghrelin and GH-releasing peptide-6 (GHRP-6) on the regulation of the functional expression of voltage-gated Na$^+$ channels using the tumoral somatotrope GC cell line as a model. Cells were cultured under control conditions or in presence of the GH secretagogues (GHS) for 96 h, and Na$^+$ currents (I\textsubscript{Na}) were characterized in whole cell patch-clamp experiments. GHS treatment significantly increased I\textsubscript{Na} density in a dose-dependent manner. The effects of GHRP-6 were accompanied by an augment in conductance without changes in the kinetics and the voltage dependence of the currents, suggesting an increase in the number of channels in the cell membrane. Sustained inhibition of L-type Ca$^{2+}$ channel activity decreased I\textsubscript{Na} density and prevented the effects of the GHS, whereas long-term exposure to an L-channel agonist increased I\textsubscript{Na} density and enhanced the actions of GHRP-6, indicating that Ca$^{2+}$ entry through these channels plays a role in the regulation of Na$^+$ channel expression. Likewise, GHRP-6 failed to enhance Na$^+$ channel expression in the presence of membrane-permeable inhibitors of protein kinases A and C, as well as the Ca$^{2+}$/calmodulin-dependent kinase II. Conversely, treatment with a CAMP analog or a protein kinase C activator enhanced both basal and GHS-induced secretion of GH measured by enzyme-linked immunosassay, suggesting that GHRP-6 acting through the ghrelin receptor and different signaling pathways enhances Na$^+$ channel membrane expression, which favors hormone release from GC somatotropes.

calcium channels; GC cells; growth hormone-releasing peptide-6; sodium channels

GROWTH HORMONE (GH) secretion by pituitary somatotropes is basically under the control of the following three hormones: GH-releasing hormone (GHRH), ghrelin, and somatostatin (SRIF). GHRH and SRIF are synthesized and released in the hypothalamus and transported to the anterior pituitary gland through the portal circulation, whereas ghrelin is predominantly produced by the stomach but has also been detected in almost all human organs and tissues examined, including the hypothalamus and the pituitary gland (20).

The physiological actions of ghrelin and related synthetic GH-releasing polypeptides (GHRPs) are initiated by the inter-action between the peptides and specific, membrane-bound, high-affinity receptors [ghrelin receptor (GhrR)] on the surface of responsive cells (4, 18, 20, 25). Although the molecular mechanisms of GH release are ill defined, recent studies have shown that GhrR-sustained activation may be linked to increased voltage-gated Ca$^{2+}$ channel surface expression, resulting in enhanced electrical and secretory activity in clonal pituitary somatotropes (11, 12).

It is well established that voltage-gated ion channels are involved in the control of the excitation-secretion coupling that leads to hormone release in different pituitary hormone-secreting cell types, including somatotropes (7, 8, 23, 27, 30, 31). This work has also evidenced the complexity in directly comparing the expression levels and the properties of the distinct classes of ionic channels from pituitary somatotropes, because of difficulties in distinguishing them from other adenohypophyseal cells in primary cultures. An alternative to cope with this inconvenience is the use of the GC cell line, which represents a homogeneous in vitro model of somatotropes (11, 12, 19).

GC somatotropes continuously release GH and behave as endogenous pacemakers. They express voltage-gated channels that work in concert to generate rhythmic spontaneous action potentials (SAP; see Refs. 11 and 19). The rising phase of SAP results mainly from Na$^+$ entry and subsequent Ca$^{2+}$ influx through L-type channels. Ca$^{2+}$-activated and delayed-rectifier K$^+$ currents repolarize the membrane to an afterdepolarization. The slow pacemaker depolarization that allows regenerative activity results from the decay of Ca$^{2+}$-activated K$^+$ currents superimposed on a Na$^+$ inward current. This depolarizes the membrane to the threshold potential for the T/R-type Ca$^{2+}$ current that activates the L-type current and initiates a new cycle (19).

Previous work from our laboratory has shown that the chronic (96-h) treatment with ghrelin or its synthetic analog GHRP-6 increases the firing frequency of SAP in the GC cell line, an effect that is associated with a significant increase in inward current density (11). At least one type of Na$^+$ and two types of Ca$^{2+}$ (L and T/R) currents have been identified in the membrane of GC somatotropes, and these determine the whole cell inward current. Likewise, the stimulatory effects of ghrelin and GHRP-6 were shown to involve an increase in Ca$^{2+}$ currents (of the L type) that alter the frequency and duration of SAP compatible with an increase in GH secretion (12). The purpose of this study was to evaluate the actions of ghrelin and GHRP-6 chronic treatment on the amplitude, time course, and voltage dependence of whole cell Na$^+$ currents (I\textsubscript{Na}) and to analyze the signaling routes employed by GHRP-6 to regulate channel activity using the tumoral somatotrope GC cell line as...
a model. Our results indicate that 1) ghrelin stimulates voltage-gated Na⁺ channel cell surface abundance; 2) the activity of L-type Ca²⁺ channels is a major determinant of the number of functional Na⁺ channels; and 3) the stimulatory effects of the GH secretagogues (GHS) on Na⁺ channels and on GH release may involve the activation of multiple signaling cascades.

**MATERIALS AND METHODS**

*Chemicals.* Ghrelin (catalog no. 55-0-03A), GHRP-6 (catalog no. 52-1-80B) and α-(Lys³)-GHRP-6 (catalog no. 52-1-83A) were purchased from American Peptide (Sunnyvale, CA). (+)-BAY K 8644 (catalog no. B-350), KT-5720 (catalog no. K-190), chelerythrine (CHE; catalog no. C-400), and tetrodotoxin (TTX; catalog no. T-550) were from Alomone Laboratories (Jerusalem, Israel). Actinomycin D (Act D; catalog no. A-1410), cycloheximide (CHX; catalog no. C-7634), KN-62 (catalog no. I-2142), and nifedipine (Nif; catalog no. N-7634) were from Sigma-Aldrich (St. Louis, MO). Dibutyryl-cAMP (DBcAMP; catalog no. CN-125) was from Biomol International (Plymouth Meeting, PA). Phorbol 12-myristate 13-acetate (PMA; catalog no. P-1039) was from A.G. Scientific (San Diego, CA). All other chemicals were of reagent grade and were purchased from different commercial sources.

*Cell culture and electrophysiology.* The GC cell line was maintained in culture as described elsewhere (11) using MegaCell DMEM culture medium (Sigma-Aldrich) supplemented with 3% FBS (MP Biomedicals, Aurora, OH), and 100 IU/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. Once a week, GC cells were harvested by trypsin-EDTA (Sigma-Aldrich) treatment (0.05% wt/vol and 453 mM, respectively) and reseeded at a density of ~2 x 10⁶ cells/flask of 25 cm². For electrophysiology, cells were seeded in 35-mm culture dishes at a density of ~7.5 x 10⁴. The culture medium (with or without peptide additions) was replenished every day up to 5 days. Control cells were incubated for 5 days in standard culture conditions without peptides and/or drugs.

Membrane capacitance was determined as described previously (1) and 5 glucose (pH 7.3). The osmolarity was adjusted to 300 and 290 mOsmol/l for the bath and pipette recording solutions, respectively. Membrane capacitance was determined as described previously (1) and used to normalize currents. Control and treated cells were rinsed with peptide-free culture medium and maintained in this medium for ~60 min before electrophysiological recording.

*Data analysis.* Data are expressed as means ± SE. Statistical significance was established using Student’s t-test (P < 0.05) when two means were compared. The significance of the differences between three or more means was assessed by one-way ANOVA according to the Scheffe test. Data points in the graph of GHRP-6 concentration vs. Iₙa density increase were fitted using a logistic equation of the form:

\[
I_{Na} = I_{max}/[1 + (EC_{50}/x)^n]
\]

where \(I_{max}\) represents the maximum Iₙa density increase, \(EC_{50}\) is the concentration of GHRP-6 required to achieve the half-maximal effect, \(x\) is the GHRP-6 concentration, and \(n\) is the slope.

Data points in the time course of loss of Na⁺ channel activity were fitted by a single exponential:

\[
I_{Na} = A\exp(-t/\tau) + c
\]

where \(A\) is the initial amplitude, \(t\) is time, \(\tau\) is the time constant for inactivation, and \(c\) is a constant.

**RESULTS**

*Chronic treatment with GHS upregulates Iₙa in GC cells.* Given that the chronic treatment with ghrelin or GHRP-6 increases the firing frequency of SAP in the GC cell line, an action that is apparently linked with an increase in macroscopic inward current density (11), we sought to determine whether the functional expression of voltage-gated Na⁺ channels is affected by GHS treatment. We first examined the effect of ghrelin treatment on the whole cell Iₙa of GC cells. As shown in Fig. 1A, left, voltage steps from a holding potential (\(V_h\)) of −80 to 0 mV evoked inward currents that reached a peak in <1 ms and decayed quickly to zero current. After 96 h of ghrelin treatment (10 nM), the average peak Iₙa amplitude was increased significantly (−2.4-fold) from a control value of 277 ± 32 to 555 ± 90 pA (Fig. 1A, right).

We next evaluated the actions of a synthetic agonist of the GhrR, GHRP-6, on channel activity. Examples of typical recordings are shown in Fig. 1B, left. To eliminate the role of the cell surface area as a variable, we divided the peak current by cell capacitance to obtain current density (see MATERIALS AND METHODS). After 96 h of chronic exposure to the GHRP-6 (100 nM), current density was increased substantially (~1.8-fold) when compared with the control cells (Fig. 1B, right). Iₙa regulation by GHS treatment was observed at almost all voltages tested (Supplemental Figs. 1 and 2 [Supplemental data for this article may be found on the American Journal of Physiology: Endocrinology and Metabolism website]). As expected, in the presence of the specific GHS receptor antagonist, α-Lys³-GHRP-6 (10 µM), the increase in Iₙa evoked in GC cells after chronic exposure to GHRP-6 was prevented (Fig. 1B, right). Last, the application of TTX (1 µM) to the bath recording solution abolished Iₙa in both control and GHS-treated cells (Fig. 1C). Given that the initial experiments confirmed that the effect of GHRP-6 on channel activity was similar to that of ghrelin, we decided to use GHRP-6 in subsequent experiments.

The time course of Iₙa stimulation by GHRP-6 (100 nM) is shown in Fig. 2A. In each cell investigated, the density of the macroscopic currents evoked by voltage steps from a \(V_h\) of −80 to 0 mV was obtained in the GHS-treated GC cells and compared with that of the untreated cells. A small increase in current density was appreciable within the first 24 h, which
became statistically significant at 48 h and tended to reach a maximum after 72 h of drug treatment. Changes in $I_{Na}$ by GHRP-6 are relative to time-matched controls. These results corroborate that GHRP-6 indeed regulates $I_{Na}$ density when applied chronically to GC cells. Interestingly, the stimulatory actions of GHRP-6 did not involve concurrent changes in current kinetics or voltage dependence of Ca$^{2+}$ channel function (Supplemental Fig. 3). Likewise, the increase in $I_{Na}$ density induced by long-term treatment with GHRP-6 was concentration dependent in the 1 nM to 1 $\mu$M range (Fig. 2B).

When the experimental data in the concentration-effect curve were fit to a logistic function (eq. 1) an $EC_{50}$ of 12.4 nM was obtained.

A possible explanation for the stimulatory effect of the GHS treatment on $I_{Na}$ density could be a decrease in the rate of internalization of Na$^{+}$/H$^{+}$ channels. To examine this, the level of Na$^{+}$ channels at the cell surface was monitored in the absence of new protein synthesis using CHX. As can be seen in Fig. 3A, CHX (35 $\mu$M) decreased peak amplitude of $I_{Na}$ (evoked by a voltage step from a $V_{h}$ of −80 to 0 mV) to ~16% in both control and GHRP-6-treated GC somatotropes after 48 h. The pattern of $I_{Na}$ decay in control cells was best fitted by a single exponential decay function (eq. 2) with a mean time constant of 26.5 h, suggesting that Na$^{+}$/H$^{+}$ channels have a mean half-life of ~18 h, a result that is in agreement with data reported previously (24). Interestingly, virtually the same results were obtained in the GC cells treated 96 h with 100 nM GHRP-6 (Fig. 3A), suggesting that the upregulation of $I_{Na}$ by chronic GHS treatment does not result from a decreased rate of Na$^{+}$/H$^{+}$ channel turnover.

To further investigate the mechanism underlying $I_{Na}$ stimulation after GHS treatment, the requirement for RNA and protein synthesis was then investigated. $I_{Na}$ from control and GHS-treated cells in the absence or presence of the transcrip-
The absence of GHRP-6 exhibited similar low Na$^+$ density in GC cells incubated for 48 h with Act D or CHX, which is consistent with the rapid turnover of Na$^+$ channels in clonal pituitary GH3 cells (24). We therefore sought to determine whether the upregulation of $I_{\text{Na}}$ density by GHS chronic treatment in GC cells was mediated by changes in Ca$^{2+}$ channel activity. To this end, cells were exposed for 96 h to the dihydropyridines BAY K 8644 (agonist) and Nif (antagonist). The concentration of these drugs was 500 nM. Superimposed representative traces are shown in Fig. 4A. Similar to the effect of the chronic treatment with GHRP-6, sustained elevation of cytosolic Ca$^{2+}$ by the L-type Ca$^{2+}$ channel activator BAY K 8644 resulted in larger $I_{\text{Na}}$ density values compared with the controls, during voltage-clamp depolarizations to 0 mV (Fig. 4B), both in the absence or the presence of 100 nM GHRP-6. In contrast, when the cells were incubated with the L-type Ca$^{2+}$ channel antagonist Nif with or without GHRP-6 treatment, $I_{\text{Na}}$ density decreased to ~60% of its control value (Fig. 4B). Together, these data indicate that the number of Na$^+$ channels in the cell membrane of GC somatotropes is dependent on the entry of Ca$^{2+}$ through channels of the L type.

Role of protein kinase activity in the GHS-induced upregulation of $I_{\text{Na}}$ in GC cells. To clarify the signal transduction pathway leading to the increase of functional voltage-gated Na$^+$ channels by long-term in vitro treatment with GHRP-6, we next investigated the effects of different protein kinase inhibitors. First, to determine whether the stimulatory actions of the GHS required an active adenylate cyclase-protein kinase A (PKA) pathway, we tested whether KT-5720, a known blocker of PKA (17, 26), could affect Na$^+$ channel functional expression. Figure 5A shows superimposed $I_{\text{Na}}$ traces recorded in GC cells in the control condition and after treatment with 500 nM KT-5720 (Fig. 5A, left). From these current traces it is apparent that the increase in whole cell $I_{\text{Na}}$ normally observed after chronic treatment with GHRP-6 is prevented by the application of the PKA inhibitor. Figure 5A, right, shows that the average maximum current density in control cells, recorded at 0 mV from a $V_m$ of −80 mV, was −25 ± 5 pA/pF and that this value increased by ~75% to −43.8 ± 5.6 pA/pF in cells cultured in the presence of GHRP-6 (100 nM). In spite of preventing the stimulatory effect of GHRP-6, the PKA inhibitor by itself had no effect on $I_{\text{Na}}$.

Ca$^{2+}$ influx through voltage-gated channels increases intracellular free Ca$^{2+}$ levels, which are linked to basal and GHS-induced GH secretion in somatotropes. These channels are activated during the cell membrane depolarization caused by activation of Na$^+$ channels and action potential firing. We therefore studied the influence of GHRP-6 on GH secretion in GC cells and its potential regulation by the PKA pathway. When the GHS was added to cell cultures, a significant increase in the GH released in medium was observed (Fig. 5B). The PKA blocker KT-5720, when given alone, significantly reduced the proportion of GH. When the cells were incubated with 5 mM DBcAMP (to stimulate PKA activity), GH release increased significantly, mimicking the effect of GHRP-6. On the other hand, when GC cells were treated with KT-5720 to downregulate PKA activity, the GHRP-6-mediated increase in hormone secretion was suppressed completely (Fig. 5B).

We next investigated the possibility of protein kinase C (PKC) involvement in the stimulatory effects of GHRP-6 on...
I_{Na} by evaluating the effects of the PKC inhibitor CHE. I_{Na} recorded in GC cells in the control condition and after treatment with 5 μM CHE showed that, in the presence of the PKC inhibitor, GHRP-6 failed to stimulate Na⁺ channel functional activity (Fig. 5C, left). Hence, averaged current density in control cells recorded at 0 mV from a V_h of −80 mV was increased significantly in GHRP-6-treated cells but remained unaffected when the GHS was applied in conjunction with...
CHE (Fig. 5C, right). Control experiments showed that the PKC inhibitor by itself did not affect $I_{Na}$.

To evaluate the participation of PKC in the signaling pathway of GHRP-6 and its impact on GH release, the effects of the PKC inhibitor CHE and the PKC activator PMA were analyzed. As can be seen in Fig. 5D, CHE (5 μM) significantly inhibited both basal and GHRP-6-induced GH secretion. In sharp contrast, hormone secretion was enhanced in the presence of the GHS and by PMA treatment (100 nM), although costimulation with PMA plus GHRP-6 did not result in significantly greater release of GH than with a single treatment alone (Fig. 5B). However, CHE treatment abolished the GHRP-6-evoked increase in hormone secretion. Taken together, these data corroborate that PKC activity is required for the GHRP-6 response in GC somatotropes.

Last, we evaluated the effects of KN-62, a putative inhibitor of the Ca$^{2+}$/calmodulin-dependent kinase II (Ca$^{2+}$/CaM-K II), on the GHRP-6-induced upregulation of voltage-gated Na$^+$ channel functional expression. As previously shown and confirmed in this series of experiments, 100 nM GHRP-6 significantly increased the peak $I_{Na}$ density. The involvement of Ca$^{2+}$/CaM-K II in this stimulatory effect is illustrated by the typical superimposed current traces shown in Fig. 6A. Application of 10 μM KN-62 prevented current density stimulation induced by GHS treatment, suggesting that Ca$^{2+}$/CaM-K II might participate in the signaling pathway that regulates the $I_{Na}$-enhancing mechanism. Consistent with this, incubation of GC cells in the presence of the Ca$^{2+}$/CaM-K II inhibitor before stimulation with the GHS inhibited the basal release of GH (Fig. 6B). On the contrary, stimulation of cells in the presence of 100 nM GHRP-6 significantly increased GH release, although coincubation with KN-62 prevented the increased GH release observed after GHS treatment (Fig. 6B).

DISCUSSION

The anterior pituitary is a very complex gland comprised of a heterogeneous population of well-differentiated secretory cell types, including somatotropes. A major obstacle to progress in the understanding of the mechanisms of GH secretion is the
lack of an ideal model for these studies. Such studies have been performed in a variety of animal models and in dispersed primary pituitary cell cultures, although some of them have been limited by the heterogeneity of anterior pituitary cell types. In addition, these cells cannot be propagated in culture, thus limiting the feasibility of many other studies. On the other hand, the availability of immortalized cell lines, such as the GC cells, have contributed to the understanding of pituitary physiology and have provided a model to study the mechanism by which the synthesis and release of GH is regulated. Although these cells may not replicate faithfully the responses of pituitary somatotropes, as cell models they provide a convenient and easily accessible system for studying the physiology of GH-secreting cells. In particular, the GC cell line has proven to be a useful model for studying electrical properties and ionic channel regulation by extracellular messengers (11, 12, 19).

Previous reports have suggested that the chronic treatment with ghrelin and GHRP-6 increases the firing frequency of SAP in GC cells that is associated with an increase in whole cell inward current density (11). Here, we show that the long-term exposure to GHRP-6 specifically increases the functional expression of voltage-gated Na\(^+\) channels, which are responsible for the spiking phase of action potentials. The membrane depolarization during SAP firing causes the subsequent activation of voltage-gated Ca\(^{2+}\) channels, increasing the concentration of cytosolic Ca\(^{2+}\) and promoting GH release. Therefore, the increase in Na\(^+\) channel number at the cell surface, together with the positive regulation in the functional expression of L-type Ca\(^{2+}\) channels observed when GC cells are chronically exposed to GHRP-6 (12), may help explain the increase in hormone secretion observed after GHS treatment. Additionally, in terms of physiological relevance, our results support the idea that the ghrelin/GHRP-6-induced increase in GH secretion may be the result of a direct stimulation on the somatotrope via phospholipase C (PLC)/PKC (as we shall discuss later), and therefore that the GHS might be acting as facilitators of the response to hypothalamic GHRH most likely as a result of increased Na\(^+\) and Ca\(^{2+}\) voltage-gated channel expression (8, 12).

Although the cellular mechanism by which chronic treatment with ghrelin and GHRP-6 increases the abundance of Na\(^+\) channels at the cell surface in GC cells has yet to be

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**Fig. 6.** Macroscopic $I_{\text{Na}}$ and GH release regulation by the Ca\(^{2+}\)/calmodulin-dependent kinase II (Ca\(^{2+}\)/CaM-K II)-dependent pathway in GC cells. A: typical whole cell Ca\(^{2+}\) currents recorded by depolarizing the membrane for 10 ms to 0 mV from a $V_m$ of −80 mV in the absence (control) and after treatment with the Ca\(^{2+}\)/CaM-K II inhibitor KN-62 alone or in combination with 100 nM GHRP-6 (left). The summary of the data with GHRP-6 and the inhibitor is shown on right. The no. of recorded cells is given in parentheses.*Significant difference ($P < 0.05$) compared with the control. $B$: average amount of GH released under control conditions and after diverse treatments, as indicated. Culture media were collected, and GH was measured by ELISA as in Fig. 5. Bars represent averaged data (+SE) from 3 independent experiments. *Significant differences ($P < 0.05$) compared with the untreated control cells.
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defined, the experiments with CHX and Act D (a RNA synthesis inhibitor) suggested that plasma membrane channel upregulation seems to result from increased transcription and protein synthesis rather than an increased half-life ($t_{1/2}$) of Na$^+$ channels.

Likewise, previous studies of our research group have shown that the entry of Ca$^{2+}$ through L-type channels has a long-term positive impact on the number of Na$^+$ channels located in the plasma membrane of GH3 cells and that prolonged changes in L channel activity lead to parallel changes in the level of $I_{\text{Na}}$ expression (24). More recently, we have also provided evidence that current density through high-voltage-activated Ca$^{2+}$ channels (mainly of the L type) in the GC cells can undergo marked changes in response to the chronic influence of ghrelin/GHRP-6 (12). Together these results suggest that Ca$^{2+}$ influx through L-type channels plays a key role in the increase in $I_{\text{Na}}$ density observed after long-term exposure to GHS. In the present report, we have tested the hypothesis that ghrelin/GHRP-6 treatment is affecting Na$^+$ channel surface expression as a consequence of increased Ca$^{2+}$ channel activity. This is consistent with the idea that changes in free Ca$^{2+}$ concentration have an effect on gene expression. Thus our present studies have identified one of the putative physiological signals that use this mechanism (enhanced Ca$^{2+}$ channel expression) to regulate Na$^+$ channel expression.

Based on the above-mentioned results, we speculate that long-term exposure to GHS might increase Ca$^{2+}$ influx in GC somatotropes and could stimulate the expression of a transcription factor(s) that promotes Na$^+$ channel synthesis or a protein that favors the insertion of preassembled Na$^+$ channels in the plasma membrane. In this regard, it is worth noting that nuclear Ca$^{2+}$ is an important regulator of gene expression following membrane depolarization of excitable cells. Hence, nuclear Ca$^{2+}$ transients in neurons activate gene transcription by a mechanism that involves the cAMP response element (CRE) and the CRE-binding protein, CREB (16). In hippocampal neurons, for example, Ca$^{2+}$ influx through L-type channels (and N-methyl-d-aspartate receptors) is capable of causing rapid translocation of Ca$^{2+}$/CaM-K II/IV to the nucleus, which is important for CREB phosphorylation (10).

Likewise, it has been established that the voltage-gated Na$^+$ channel consists of a highly glycosylated pore-forming $\alpha$-subunit ($\sim$260 kDa) with associated auxiliary $\beta$-subunits (of $\sim$33–36 kDa). The $\alpha$-subunit alone is capable of forming a functional ion-selective channel. In mammals, nine $\alpha$ (Nav1.1-Nav1.9)- and four $\beta$ (Nav$\beta_1$-Nav$\beta_3$)- subunit genes have been identified (5, 9). Interestingly, the covalent association of neuronal Na$^+$ channels with the Nav$\beta_2$ subunit seems to be critical for high-level surface expression. In neurons, $\sim$70% of Na$^+$ channels appear to lack associated Nav$\beta_2$ subunit and account for a substantial intracellular pool, exit from which is likely to be the rate-limiting step in channel trafficking (9). Therefore, measurement of the cell abundance of Na$^+$ channel subunits after treatment will be important to begin to understand the stimulatory actions of GHS on somatotrope’s excitability and hormone secretion.

The actions of GHS treatment on pituitary somatotropes are linked to G protein-coupled GhrR (6), and GHS-stimulated GH release depends on the cAMP/PKA and PLC/PKC systems and extracellular Ca$^{2+}$ influx (3, 13, 22, 33, 34). In the present study, two specific inhibitors of PKA and PKC (KT-5720 and CHE, respectively) were found to have an effect on the $I_{\text{Na}}$ response to GHRP-6 and on GH secretion from GC cells, suggesting the involvement of these protein kinase systems. Synergistic PKA and PKC regulation of Na$^+$ channels might be important in somatotropes subjected to particular stimulatory signals. Upregulation of $I_{\text{Na}}$ by PKA and PKC should decrease the voltage thresholds required for SAP firing, and a weaker depolarization should be needed to elicit an exocytotic response. However, it should be noted that the ultimate response of GH-secreting cells to GHS that stimulate the PKC and/or PKA pathway depends on the global interplay of multiple proteins.

Interestingly, pharmacological agents that stimulate PKA activity can enhance $I_{\text{Na}}$ density in cardiac myocytes, suggesting an increase in Na$^+$ channel expression at the plasma membrane (14, 21), and activation of PKA stimulates the trafficking of Nav1.5 to the cell surface (35). The $\alpha$-subunit of Nav1.5 contains three consensus PKA phosphorylation sites in the intracellular loop connecting domains I and II. This motif has been shown to act as an endoplasmic reticulum retention signal in other membrane proteins, and its deletion in Nav1.5 abolishes the PKA-dependent trafficking to the cell surface (34). Further experiments are needed to determine whether the Na$^+$ channels expressed in GC cells contain this functional domain.

Likewise, it is well known that various kinases are capable of phosphorylating CREB, including Ca$^{2+}$/CaM-K II/IV (28, 29). Another novel finding of our work is that the inhibitor of Ca$^{2+}$/CaM-K II, KN-62, abolished the GHS-induced increase in Na$^+$ channel density and GH secretion in GC cells. These results suggest that Ca$^{2+}$/CaM-K II plays a role in the signaling pathway from GhrR activation to an increase in Na$^+$ channel functional expression. Interestingly, phosphorylation of CREB on Ser133 and Ser142 by nuclear Ca$^{2+}$/CaM-K II, Ser133, and Ser142 by nuclear Ca$^{2+}$/CaM-K II promotes the activity of genes containing an upstream cAMP-responsive element (2). Phosphorylation on Ser142 is believed to block the Ser133 phosphorylation-dependent activation of CREB, suggesting a possible mechanism for the regulation of CREB activity (28). Extensive work on the promoter region of the Na$^+$ channels expressed in GC cells will be needed to define the role of nuclear Ca$^{2+}$ and Ca$^{2+}$/CaM-K II on intracellular protein cascades leading to activation of gene transcription after GHS treatment.

In summary, this study demonstrates that the chronic stimulation of the GHS receptor with ghrelin or GHRP-6 increases the abundance of voltage-gated Na$^+$ channels at the cell surface of rat tumor GC cells through the activation of multiple signaling cascades, including the cAMP-PKA and PLC-PKC pathways as well as extracellular Ca$^{2+}$-dependent mechanisms.

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