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Ghrelin Reduces Voltage-Gated Potassium Currents in GH_3 Cells Via Cyclic GMP Pathways

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Ghrelin is an endogenous growth hormone secretagogue (GHS) causing release of GH from pituitary somatotropes through the GHS receptor. Secretion of GH is linked directly to intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), which is determined by Ca^{2+} influx and release from intracellular Ca^{2+} storage sites. Ca^{2+} influx is via voltage-gated Ca^{2+} channels, which are activated by cell depolarization. Membrane potential is mainly determined by transmembrane K^+ channels. The present study investigates the in vitro effect of ghrelin on membrane voltage-gated K^+ channels in the GH_3 rat somatotrope cell line. Nystatin-perforated patch clamp recording was used to record K^+ currents under voltage-clamp conditions. In the presence of Co^{2+} (1 m*M*, Ca^{2+} channel blocker) and tetrodotoxin (1 µ*M*, Na^+ channel blocker) in the bath solution, two types of voltage-gated K^+ currents were characterized on the basis of their biophysical kinetics and pharmacological properties. We observed that transient K^+ current (I_A) represented a significant proportion of total K^+ currents in some cells, whereas delayed rectifier K^+ current (I_K) existed in all cells. The application of ghrelin (10 n*M*) reversibly and significantly decreased the amplitude of both I_A and I_K currents to 48% and 64% of control, respectively. Application of apamin (1 µ*M*, SK channel blocker) or charybdotoxin (1 µ*M*, BK channel blocker) did not alter the K^+ current or the response to ghrelin. The ghrelin-induced reduction in K^+ currents was not affected by PKC and PKA inhibitors. KT5823, a specific PKG inhibitor, totally abolished the K^+ current response to ghrelin. These results suggest that ghrelin-induced reduction of voltage-gated K^+ currents in GH_3 cells is mediated through a PKG-dependent pathway. A decrease in voltage-gated K^+ currents may increase the frequency, duration, and amplitude of action potentials and contribute to GH secretion from somatotropes.

Key Words: Ghrelin; GH_3 cells; voltage-gated K^+ channels.

Introduction

Ghrelin, a 28-amino-acid peptide first discovered in the stomach, activates the growth hormone secretagogue (GHS) receptor and stimulates GH secretion both in vivo and in vitro in several species *(1,2)*. Ghrelin stimulates GH secretion from somatotropes in vitro through the activation of multiple signaling cascades, including phospholipase C (PLC) /PKC, cAMP/PKA, and through intra- and extracellular Ca^{2+}-dependent mechanisms *(3)*. In addition, the Ca^{2+} influx through L-type voltage-gated Ca^{2+} channels and Na^+ influx through Na^+ channels are involved in the action of ghrelin on somatotropes *(4)*. The mechanism underlying the ghrelin-induced membrane depolarization, leading to the opening of voltage-gated Ca^{2+} and Na^+ channels, is still not clear. Somatotropes, like most pituitary endocrine cells, are excitable cells with irregular spontaneous action potentials *(5)*. Cell excitabilitydepends on the opening and closing of transmembrane ion channels and the level of resting membrane potential. The K^+ conductance across the membrane is responsible for the resting potential *(6)*. It is reported that somatostatin can increase several types of K^+ currents, including voltage-gated K^+ currents and inward rectifying K^+ currents in rat, ovine, and human somatotropes, to hyperpolarize cell membrane potential *(7–10)*. GHRH depolarizes the cell membrane potential of somatotropes and reduces voltage-gated K^+ currents in GH4C1 cells and human pituitary adenoma cells *(11–13)*. GH-releasing peptide-6 (GHRP-6), a synthetic GH secretagogue, decreases transient and delayed rectifying K^+ currents in rat somatotropes through the GHS receptor *(14)*. This investigation aims to test the effect of ghrelin on the voltage-gated K^+ currents in the GH_3 cell line, which is derived from a rat pituitary tumour secreting GH and expressing the GHS receptor (GHS-R).

Results

Characterization of the Voltage-Gated K^+ Currents

K^+ currents were stimulated by voltage steps from a holding potential (hp) of −80 mV. Current was measured in the range from −50 mV to +60 mV, with an increment of 10 mV between steps (Fig. 1Aa). In order to characterize the possible subtypes of voltage-gated K^+ currents, we also used

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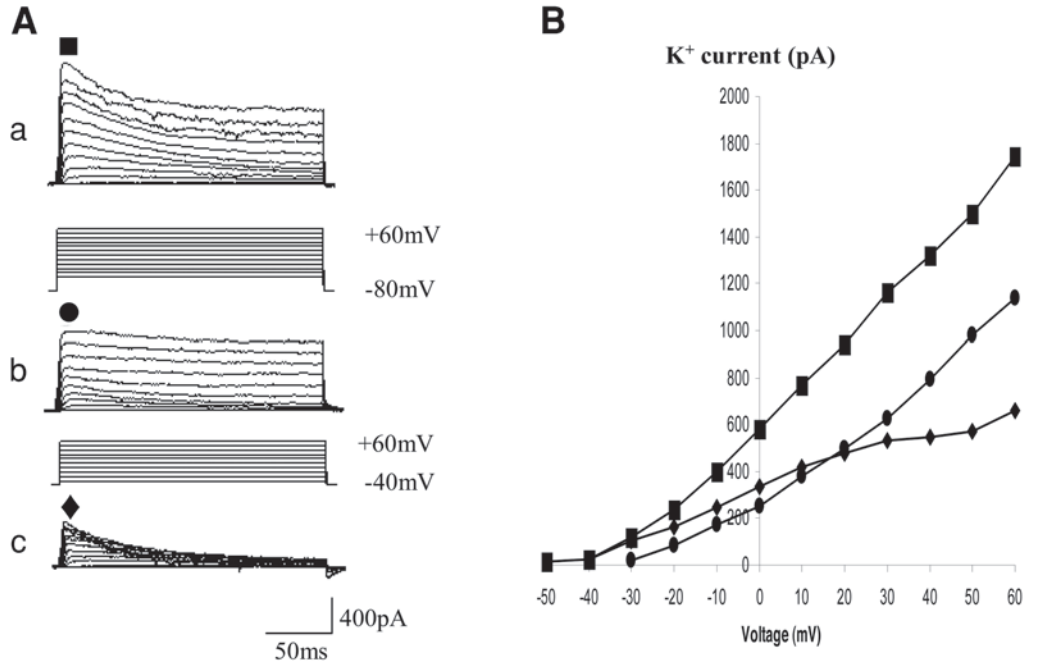

Fig. 1. Characterization of the voltage-gated K currents. (**A**) The K^+ current following voltage steps from a holding potential of (**a**) –80 mV and (**b**) –40 mV and stepped to test pulses in increments of 10 mV to +60 mV as shown inthe pulse protocols. (**c**) The difference in K^+ current between two different holding potentials (**a–b**). (**B**) Current-voltage relationships of the K^+ currents shown in panel **A** with a holding potential of –80 mV (■), –40 mV (●) and with substraction of traces in Ab from traces in Aa (◆) measured at the peak of each current trace.

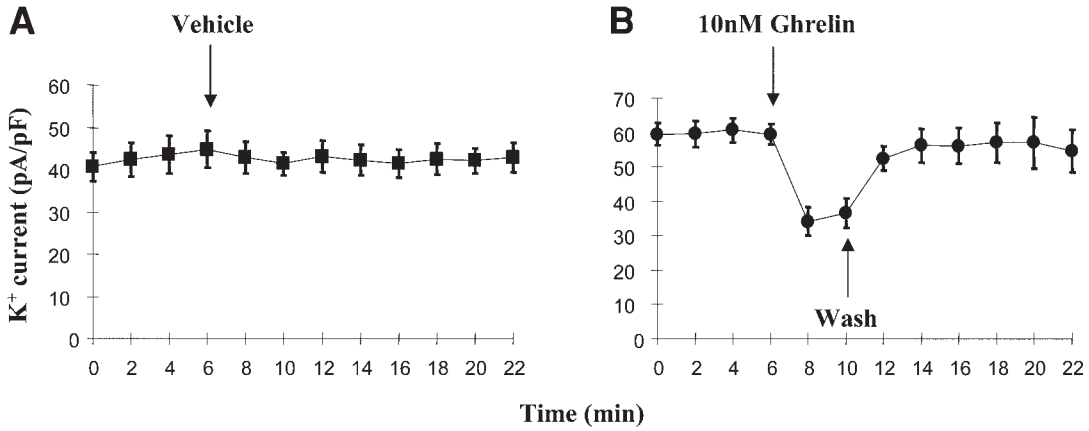

Fig. 2. Time-response relationship of the effect of ghrelin on voltage-gated K^+ currents. Voltage-gated K^+ currents were recorded every 2 min with a depolarizing pulse to +60 mV from a holding potential of –80 mV. Vehicle (recording medium, **A**) or ghrelin (10 n*M*, **B**) was applied to cells as indicated by the arrow. Data are shown as mean ± SEM (n = 5) of the peak current in each trace shown.

a hp of –40 mV (Fig. 1Ab) to record delayed rectifier K^+ currents (I_K) alone as I_A channels are closed at this hp. There was significant difference in the kinetics obtained at two different hps. We isolated transient K^+ current (I_A) by subtracting the I_K current obtained at the hp of –40 mV from the total currents obtained at the hp of –80 mV (Fig. 1Ac). Total, I_K, and I_A K^+ currents were measured at the peak of each current trace (Fig. 1B). Similar results were obtained in a group of five cells.

Effect of Ghrelin on Voltage-Gated K+ Currents

The effect of local application of ghrelin (10 n*M*) on voltage-gated K^+ currents was examined using a hp of –80 mV. Application of ghrelin evoked a significant reduction in the K^+ current amplitude recorded with a 200 ms depolarizing pulse to +60 mV. Figure 2 shows the mean ± SEM values of the peak current measured from five recorded cells. We use normal bath solution as vehicle to show the stable total K^+ currents under control conditions with vehicle

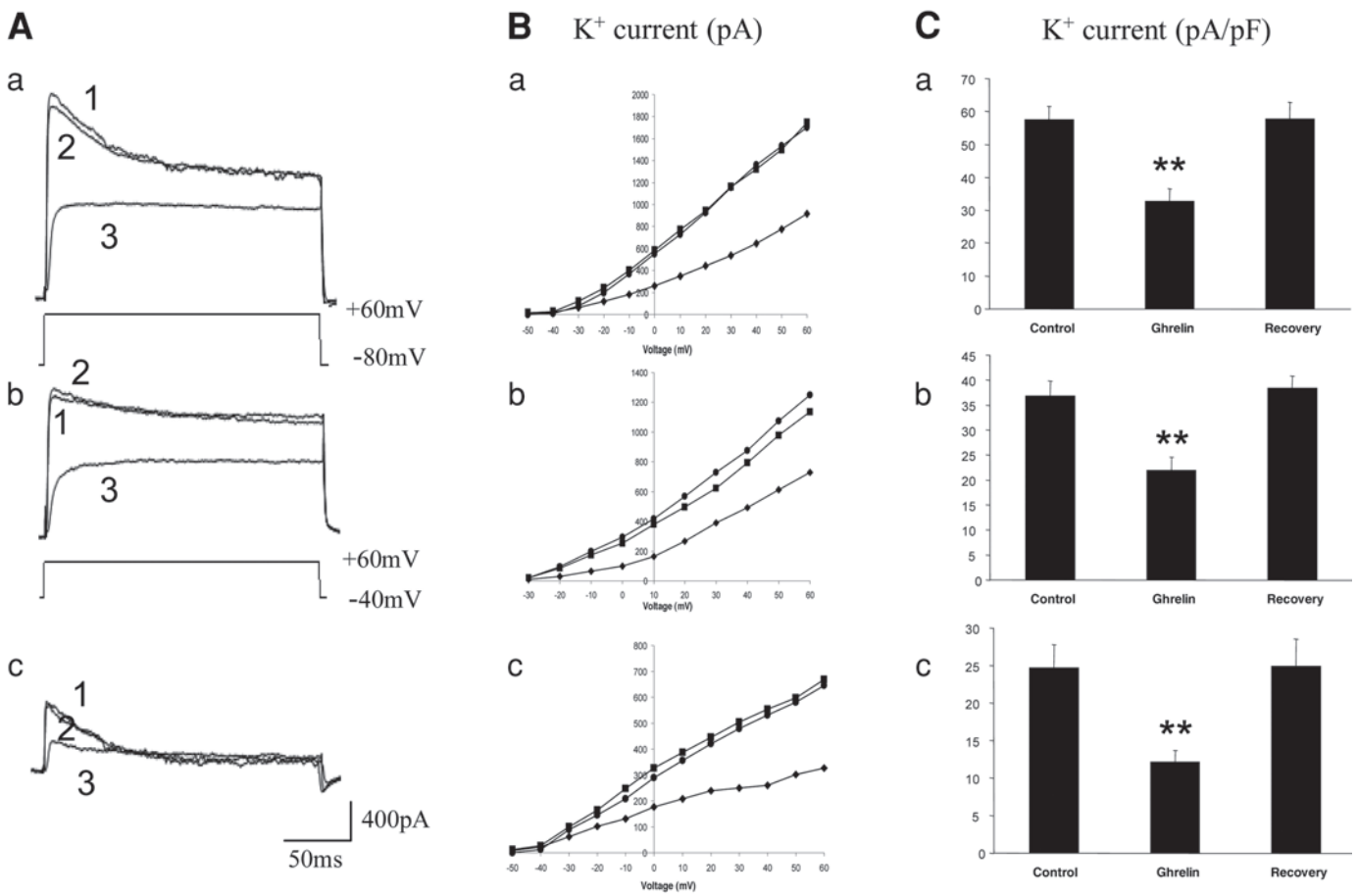

Fig. 3. Effect of ghrelin on voltage-gated K^+ currents. (**A**) Representative voltage-gated K^+ currents of GH_3 cell at +60 mV evoked from a holding potential of (**a**) −80 mV and (**b**) −40 mV. Difference in K^+ current traces between two different holding potentials highlights I_A current only. Numbers indicate control (1), during application of 10 n*M* ghrelin (3), and 2 min after the removal of ghrelin (2). (**B**) Current–voltage relationships of the peak and steady-state K^+ currents recorded with a holding potential of (**a**) −80 and (**b**) −40 mV, respectively, stepped to the voltages indicated. Data were obtained from the same cell as in **A**. (**c**) Comparison of peak K^+ currents at two different holding potentials. In all *I-V* plots, the symbols represent control (■), during application of ghrelin (◆), and 2 min after the removal of ghrelin (●). (**C**) Results (mean ± SEM, $n = 5$) for the peak K^+ current measured following a depolarizing pulse to +60 mV from a holding potential of (**a**) −80 mV, (**b**) −40 mV, and (**c**) the difference in K^+ current between the two different holding potentials. Ghrelin (10 n*M*) significantly (**$p < 0.01$) reduced the amplitude of the K^+ current with a full recovery 2 min after removal of ghrelin in **a**, **b**, and **c**.

application (Fig. 2A). The effect of ghrelin occurred immediately, causing maximal reduction of the peak current within 2 min, and the amplitude of the peak current recovered completely by 4 min after the removal of ghrelin (Fig. 2B).

In the presence of ghrelin, reduction of K^+ currents was maintained. Ghrelin was then applied to the bath solution using the perfusion system, which gave an accurate and stable concentration of ghrelin in bath solution with a possibility to include reagents affecting intracellular signaling systems. Perfusion system was then used in all following experiments. The current–voltage relationships and treatment of cells with different signaling regulating agents were studied using this perfusion drug delivery system. For the effect of ghrelin on different types of K^+ currents, hp of −80 mV and −40 mV were used to isolate I_A and I_K. Application of ghrelin (100 n*M*) significantly reduced both I_A and I_K currents following the application of depolarizing pulses up to +60 mV under two hps; 100 n*M* and 10 n*M* doses of ghrelin had the same effect on K^+ currents, while 1 n*M* had no effect (data not shown). Therefore, the concentration of ghrelin used in all subsequent experiments was 10^{-8} *M*. The traces in Fig. 3A show the typical response to 10 n*M* ghrelin and the recovery of K^+ current upon washout recorded with a hp of −80 mV (a), −40 mV (b), and the isolated I_A current (c = a–b) at a depolarizing potential of +60 mV. Current–voltage relationships were obtained from the same cell as that recorded in panel A, with depolarizing pulses ranging from −50 to +60 mV at hp of −80 mV, and −30 to +60 mV at hp of −40 mV (Fig. 3B). From a group of five cells, the reduction in the K^+ current at +60 mV was statistically significant ($p < 0.01$) for total, I_K, and I_A currents (Fig. 3C).

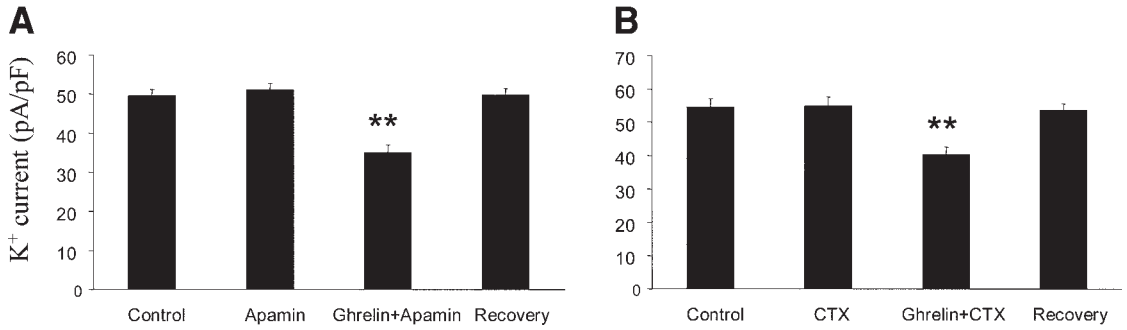

Fig. 4. Blockade of SK and BK channels does not alter the ghrelin-induced reduction in K^+ current. K^+ current at +60 mV (mean ± SEM, $n = 4$) from a holding potential of −80 mV is still significantly (**$p < 0.01$) reduced by ghrelin in the presence of either (**A**) apamin (1 μ*M*) or (**B**) charybdotoxin (CTX, 1 μ*M*).

Effect of Apamin and Charybdotoxin on Ghrelin-Induced K^+ Current Reduction

Apamin is a specific blocker of the small conductance Ca^{2+}-activated K^+ (I_{KCa}) channels known as SK channels. Doses up to 1 μ*M* did not modify the voltage-gated K^+ currents in our recording conditions in the presence of 1 m*M* Co^{2+}. Furthermore, apamin did not affect the K^+ current response to ghrelin ($p < 0.01$) recorded with hp of −80 mV and depolarizing pulse to +60 mV (Fig. 4A), suggesting that SK channels are unlikely to be involved in the K^+ current response to ghrelin under this recording condition.

Charybdotoxin (1 μ*M*), a specific blocker of large conductance I_{KCa} (BK channels), did not alter the K^+ current size or the response to ghrelin ($p < 0.01$), recorded with hp of −80 mV and depolarizing pulse to +60 mV (Fig. 4B), suggesting that BK channels are unlikely to be involved in the K^+ current response to ghrelin under this recording condition.

Involvement of cAMP/PKA System in the K^+ Current Response to Ghrelin

Rp-cAMP is a membrane-permeable cAMP antagonist. Incubation of cells with Rp-cAMP (100 μ*M*) for 10 min did not alter the K^+ current or the response to ghrelin ($p < 0.01$) recorded with hp of −80 mV and depolarizing pulse to +60 mV (Fig. 5A, $n = 5$). H_{89} (1 μ*M*), a selective PKA inhibitor, did not modify the voltage-gated K^+ currents when applied alone. Furthermore, H_{89} did not affect the K^+ current response to ghrelin ($p < 0.01$) recorded with hp of −80 mV and depolarizing pulse to +60 mV (Fig. 5B, $n = 5$). Doses for Rp-cAMP and H_{89} were determined based on previous published work *(11,12)*.

Involvement of the PKC System in the K^+ Current Response to Ghrelin

Calphostin C and chelerythrine are specific blockers of PKC. Incubation with either calphostin C (1 μ*M*) or chelerythrine (1 μ*M*) for 10 min did not significantly change the voltage-gated K^+ current or its response to ghrelin (Figs. 5C, D; $n = 5$). Doses for calphostin C and chelerythrine were determined based on previous published work *(11,12)*.

Involvement of cGMP/PKG System in the K^+ Current Response to Ghrelin

KT5823 is a specific blocker of cGMP-dependent PKG. Incubation with KT5823 (1 μ*M*) for 10 min did not significantly change the voltage-gated K^+ current. The K^+ current response to ghrelin was, however, totally abolished ($n = 5$) when the PKG system was inhibited (Fig. 6). Dose for KT5823 was determined based on published work *(44,45)*.

Discussion

The present study was undertaken to examine the effect of ghrelin on K^+ channel function in GH_3 cells. The results indicate that voltage-gated K^+ currents (both I_A and I_K current) are rapidly and reversibly reduced by application of ghrelin to GH_3 cells. The effect of ghrelin does not act upon either BK or SK channels and is independent of both the cAMP/PKA system and the PKC system. This reduction of voltage-gated K^+ currents induced by ghrelin appears to be mediated by the cGMP/PKG system in GH_3 cells.

GH is an anabolic hormone that regulates growth and development *(15)*. It is well established that GH secretion is under the reciprocal control of GHRH and SRIF at the level of the pituitary gland with additional regulation by ghrelin *(1,16)*. GH release is stimulated by GHRH and inhibited by SRIF *(16)*. Ghrelin, as an endogenous ligand for GHS-R, seems to be involved in an additional neuroendocrine pathway for GH control *(1)*. Indeed ghrelin strongly stimulates GH release, both in vivo and in vitro, in a wide range of species including human and rodents *(1,2)*. The findings that ghrelin mRNA and peptide have been detected in the rat and human pituitary suggest that ghrelin may influence the GH secretion in a paracrine and autocrine manner *(17,18)*. GHRH has been suggested to regulate the level of ghrelin in the pituitary *(18,19)*.The effect of ghrelin on GH release is dose- and time-dependent and SRIF can antagonize the action of ghrelin at the level of the pituitary gland *(2,20)*. The effect of ghrelin on GH secretion is thought to be linked to multiple signaling pathways. In porcine somatotropes, GH release in response to ghrelin depends on the cAMP/PKA and PLC/PKC systems and through extracel-

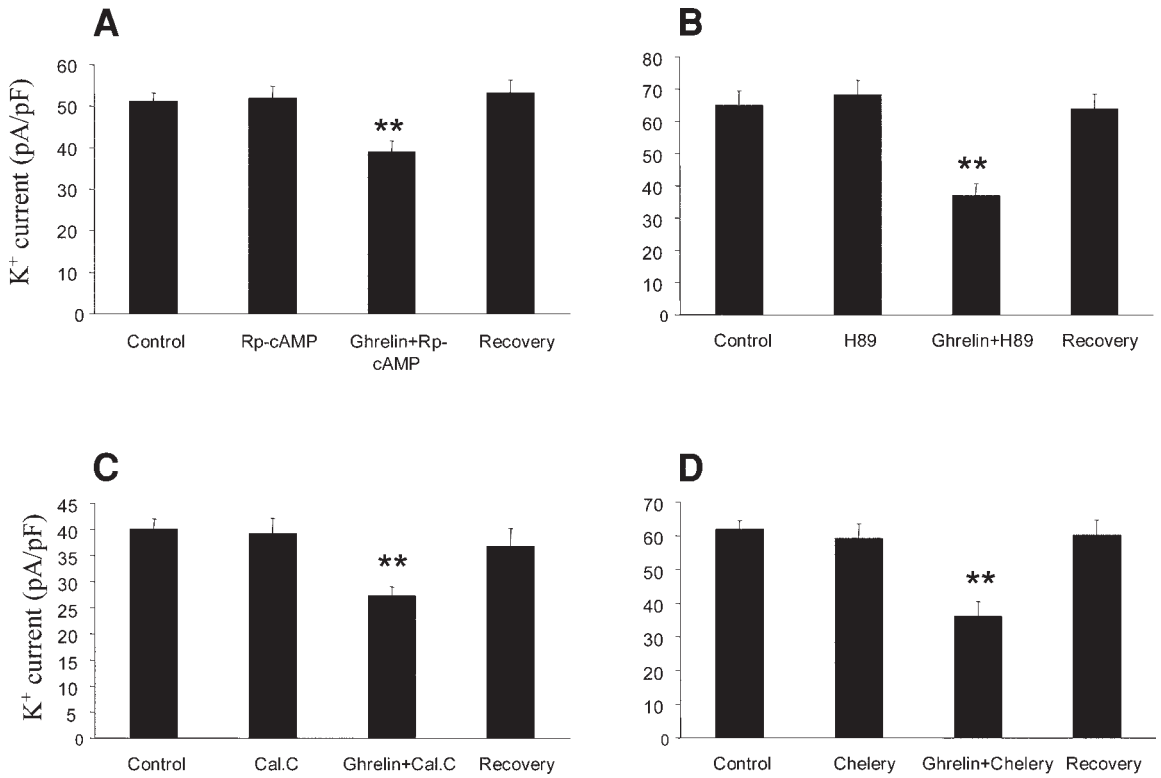

Fig. 5. Antagonists of cAMP-PKA or PKC systems have no effect on the ghrelin-induced reduction in K^+ current. Peak K^+ current (mean ± SEM, $n = 5$) evoked by depolarizing pulses from a holding potential of −80 mV to +60 mV. The application of (**A**) Rp-cAMP (100 μ*M*), (**B**) H_{89} (1 μ*M*), (**C**) Calphostin C (Cal C, 1 μ*M*), or (**D**) chelerythrine (Chelery, 1 μ*M*) did not modify the recorded K^+ current amplitude. Ghrelin significantly (**$p < 0.01$) and reversibly reduced the amplitude of K^+ currents in the presence of any of these compounds.

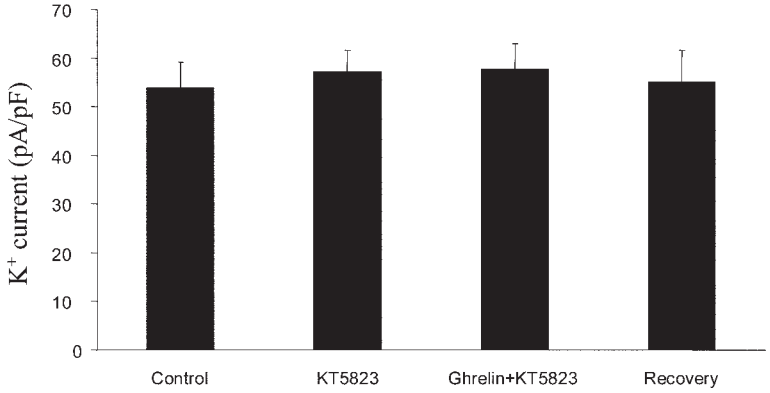

Fig. 6. Effect of blockade of cGMP/PKC systems on the ghrelin-induced reduction in the K^+ current. Peak K^+ current (mean ± SEM, $n = 5$) evoked by depolarizing pulses from a holding potential of −80 mV to +60 mV. The application of KT5823 (1 μ*M*) did not modify the recorded K^+ current. Ghrelin application in presence of the KT5823 did not reduce the amplitude of the K^+ current, nor did the washout of these compounds.

lular Ca^{2+} influx *(3)*. Ghrelin action on GH release from isolated rat anterior pituitary cells is achieved through both intracellular Ca^{2+} release and extracellular Ca^{2+} influx. Furthermore, the L-type voltage-gated Ca^{2+} channel and Na^+ influx are related to the extracellular Ca^{2+} influx induced by ghrelin *(4)*.

Somatotrope excitability depends on the properties of transmembrane ion channels and the level of resting membrane potential. The K^+ current across the membrane is mainly responsible for the resting potential, although other ions such as Na^+ and Ca^{2+} may participate *(6)*. Based on differential time- and voltage-dependent properties and pharmacological sensitivities, several kinds of voltage-gated K^+ currents have been identified in pituitary endocrine cells. These K^+ currents include four major types: inward rectifying (I_{IR}), transient outward (I_A), delayed rectifying outward (I_K), and M-type currents *(5,13,21)*. Both I_A and I_K have been demonstrated to be involved in membrane elec-

trical activity of somatotropes *(8)*. I_A is thought to be partially responsible for maintaining the resting potential levels and to participate in the repolarizing process of action potentials *(5)*. I_K current can be activated by a group of continuously firing action potentials, which may assist the cell in producing subsequent action potentials and also prevent overloading of free Ca^{2+} in the cell *(22)*. SRIF increases both voltage-gated and inwardly rectifying K^+ currents in rat, ovine, and human somatotropes *(7–10)*. GHRH decrease voltage-gated K^+ current in human pituitary adenoma cells and GH4C1 cells *(11,12)*. GHRP-6 decreases transient and delayed rectifying K^+ current in rat somatotropes *(14)*. However, GHRP-2 increases voltage-gated K^+ current (both I_A and I_K) through an increase in the synthesis of K^+ channel protein *(23)*. Only a few reports show the effect of ghrelin on ion channels. Ghrelin inhibits G protein–coupled inward rectifier K^+ channels (Kir3) of tuberomammillary nucleus neurons *(24)*. Here we report that ghrelin reduces voltage-gated K^+ currents in the rat somatotrope GH_3 cell line. This effect of ghrelin likely occurs through the activation of GHS-R, as the GHRH-R is not expressed in GH_3 cells *(17, 25)*. GH_3 cells also express several types of K^+ channels including voltage-gated K^+ channels, inward rectifying K^+ channels, and Ca^{2+}-activated K^+ channels *(26–28)*.

Both I_A and I_K currents have previously been observed in GH_3 cells *(29)*. The proportion of each type of current that exists in a particular type of cell differs from species to species. For example, I_A makes up a large proportion of the total K^+ currents in rat pituitary cells but only accounts for a small component in sheep somatotropes *(5,8)*. In this current report using GH_3 cells, both I_K and I_A currents were found to be present. There has been no previous report of ghrelin-induced modification of either voltage-gated K^+ current. The results of the present study indicate for the first time that voltage-gated K^+ currents (both I_A and I_K current) are reversibly and significantly reduced by the direct action of ghrelin on GH_3 cells.

I_{KCa} current has been reported in pituitary endocrine cells including rat GH_3 cells and may play a role in determining resting membrane potential or pacemaker activity of cells *(26,27,30–32)*. In this experiment we used Co^{2+} to block the calcium channels. Under such a recording condition, neither charybdotoxin (BK I_{KCa} channel blocker) nor apamin (SK I_{KCa} channel blocker) affected the total recorded K^+ currents and the K^+current response to ghrelin. The involvement of I_{KCa} in the recorded effect of ghrelin on K^+ currents in this report is therefore excluded. Whether ghrelin also modifies the I_{KCa} is still an open question and needs further investigation.

The action of ghrelin or synthetic GH secretagogues (GHS) on pituitary somatotropes is linked to G protein–coupled GHS receptors (GHS-R) *(1)*. GHS-stimulated GH secretion is medi-ated by the PLC/PKC system and Ca^{2+} influx in somatotropes *(3)*. GHRP-2, a synthetic peptide GHS, reduces the inward rectifying K^+ current through the cAMP-PKA system in ovine somatotropes *(33)*. Our investigations in this report show that the cAMP/PKA system is not involved in the ghrelin-induced reduction in voltage-gated K^+ currents in GH_3 cells. It is known that the PLC/PKC system is involved in the action of GHS and ghrelin on GH secretion in somatotropes *(3,4)* and the PKC system is also involved in the action of GHRH on voltage-gated K^+ current in human pituitary adenoma cells and GH4C1 cells *(11,12)*. GHRP-2 long-term treatment in vitro increases voltage-gated K^+ current through the PKC system in ovine somatotropes *(23)*. In the present study, two specific PKC inhibitors (calphostin C and chelerythrine) were found to have no effect on the K^+ current response to ghrelin, excluding the involvement of the PKC system in the ghrelin-induced reduction in voltage-gated K^+ currents in GH_3 cells.

In addition to the cAMP/PKA and PLC/PKC systems, the cGMP-dependent protein kinase (PKGs) system also plays a potential role in regulating GH secretion from somatotropes *(34,35)*. Somatostatin inhibits cholera toxin–induced bovine GH secretion by preventing activation of the secretory process through either cyclic AMP or cyclic GMP *(36)*. GHRH can induce a robust increase in cGMP production via an extracellular Ca^{2+}- and NO-independent mechanism *(37)*. Eight-bromo-cGMP, a cell-permeable agonist of cGMP, stimulated a fourfold increase in GH release from rat pituitary cells *(35)*. Application of 8-bromo-cGMP did not, however, affect GHRH-induced GH secretion in rat pituitary cells *(38)*. C-type natriuretic peptide (CNP) stimulates secretion of GH from GH_3 cells via a cyclic-GMP-mediated pathway *(34)*. NO donors *S*-nitroso-*N*-acetylpenicillamine and sodium nitroprusside (SNP), as well as a GMP analog (dibutyryl guanosine 3'-5'-cyclic monophosphate), significantly increase GH secretion from cultured goldfish pituitary cells *(39)*. Cyclic GMP may directly modulate ion channels or indirectly act via PKG activation *(40–43)*. The specific PKG blocker, KT5823 *(44,45)*, has been demonstrated to have no effect on the recorded K^+ currents but totally abolished the K^+ current response to ghrelin. The reduction of voltage-gated K^+ currents induced by ghrelin is therefore mediated by the cGMP/PKG system in GH_3 cells.

In summary, we have demonstrated in GH_3 cells that ghrelin decreases transmembrane voltage-gated K^+ currents through the cGMP/PKG signaling pathway.This decrease in currents may increase the frequency, duration, and amplitude of action potentials and slow repolarization to increase Ca^{2+} influx via voltage-gated Ca^{2+} channels. This mechanism may contribute to the ghrelin-triggered hormone secretion from somatotropes.

Materials and Methods

Chemicals

DMEM medium powder, F12 powder, HEPES, and sodium bicarbonate ($NaHCO_3$) solution were purchased from Thermo Electron Corporation (Melbourne, Australia). Peni-

cillin–streptomycin antibiotic solution and trypsin–EDTA solution were from Gibco (Gaithersburg, MD, USA). Fetal calf serum (FCS) was purchased from Thermo Trace Ltd. (Melbourne, Australia). Tetrodotoxin (TTX) and KT5823 were purchased from Alomone Laboratories (Jerusalem, Israel). Nifedipine, nystatin, dimethyl sulfoxide (DMSO) and all general salts for recording solutions were purchased from Sigma (St. Louis, MO, USA). Ghrelin was obtained from Auspep (Parkville, Australia). Calphostin C was obtained from Merck (Kilsyth, Australia). Chelerythrine chloride, Rp-cAMP, and H_{89} were purchased from Calbiochem-Novabiochem Pty Ltd. (Alexandria, NSW, Australia).

Cell Culture and Preparation

The GH_3 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and grown as monolayer in 80 cm^2 surface area plastic disposable culture flasks (Nunc, Roskilde, Denmark) with culture medium at 37°C in a humidified atmosphere (95% air–5% CO_2). The culture medium contained 45% DMEM, 45% F12, 10% FCS, and 1% (v/v) penicillin–streptomycin antibiotic solution. Cells were harvested during the logarithmic phase of growth, at which time they were visibly confluent in the flasks. Cell suspensions were obtained by removing culture medium and adding 3 mL trypsin–EDTA solution to the flasks. The cells were then detached from the surface of the flasks and 10 mL of DMEM medium was added to cancel the trypsin activity. The cells were gently washed from the flasks and centrifuged (200*g* for 5 min). The supernatant was decanted and the pellet of cells resuspended in culture medium and seeded into 35 mm culture dishes. The medium was changed twice a week, and the electrophysiological recordings were performed after 2–5 d in culture dishes.

Electrophysiological Recording

In patch clamp experiments, the normal bath solution was composed of (m*M*): 140 NaCl, 5 KCl, 0.5 $CaCl_2$, 0.5 $MgCl_2$, 0.001 TTX, 1 $CoCl_2$, 10 glucose, and 10 HEPES at pH 7.4 and osmolarity of 310 mOsm. The tip of the pipet was filled with a solution containing (in m*M*): 55 KCl, 75 K_2SO_4, 8 $MgSO_4$, and 10 HEPES at pH 7.4 and osmolarity of 300 mOsm, and the electrode was backfilled with this solution containing nystatin (300 μg/mL in 0.1% DMSO). This concentration of DMSO alone had no effect on membrane conductance when applied to the cells.

After obtaining a high-resistance seal, the pipet potential was set to –80 mV, and voltage pulses (20 mV amplitude, 200 ms duration) were delivered periodically to monitor the access resistance. Access to the cell interior was confirmed by the appearance of a membrane capacitance transient current, which usually occurred within 3–5 min under our experimental conditions. Typically, whole cell capacitance and series resistance (using only cells with <35 MΩ) were compensated (85%) and leak current was routinely subtracted using Clampex 7.0 (Axon Instrument, USA). Axon Instrument pClamp 7.0 software was used to acquire and analyze the data. Cell culture dishes were placed on the stage of an Olympus inverted microscope. Ghrelin and drugs were applied by hand to the culture dishes during recordings. Control studies were performed by applying vehicle instead of ghrelin to the cells and this caused no change in K^+ currents. Time-response curves indicate that, in our experimental system, the maximum effect of ghrelin occurred within 2 min. A gravity pressure system was used to perfuse the cells at a rate of 1 mL/min after this time to wash off the applied drugs. All experiments were performed at room temperature (20–22°C).

Statistical Analysis

Student's paired *t* test was used to evaluate the statistical significance of differences between the means of the different groups. Group data represent at least five recordings under the same experimental conditions and are expressed as mean ± SEM.

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