Activation of Growth Hormone Secretagogue Type 1a Receptor Inhibits T-type Ca^{2+} Channel Currents through Pertussis Toxin-sensitive Novel Protein Kinase C Pathway in Mouse Spermatogenic Cells

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Key Words

Ghrelin • T-type Ca^{2+} channels • Spermatogenic cells
• Acrosome reaction

Abstract

Ghrelin, a newly isolated brain-gut peptide, has been found to play important roles in the male reproduction. However, to date, the detailed mechanisms still remain unknown. In this study, we identified a novel functional role of ghrelin in modulating T-type Ca^{2+} channel currents (T-currents) in mouse spermatogenic cells. We found that ghrelin inhibited T-currents in a dose-dependent manner. Ghrelin at 0.1 μM reversibly inhibited T-currents by ~31.7%. This inhibitory effect was blocked by D-Lys3-GHRP-6, a selective growth hormone secretagogue receptor 1a (GHS-R1a) antagonist. Intracellular infusion of GDP-b-S or pretreatment of the cells with pertussis toxin (PTX) completely blocked the inhibitory effects of ghrelin. Furthermore, ghrelin responses were abolished by the phospholipase C inhibitor U73122, but not the inactive analogue U73343. The classical and novel protein kinase C antagonist chelerythrine chlorid or GF109203X abolished

ghrelin responses, whereas Ro31-8820, a classical PKC antagonist or PKI 6-22, a PKA antagonist, elicited no such effects. Taken together, these results suggest that ghrelin acting through GHS-R1a inhibits T-currents via a PTX-sensitive novel PKC pathway in mouse spermatogenic cells, which could contribute to its male reproductive functions such as acrosome reactions.

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Introduction

Ghrelin, a newly isolated 28 amino acid brain-gastric peptide, was originally isolated from the stomach and has receptors expressed in brain and peripheral tissues including heart and pancreas [1, 2]. It has a number of physiological properties, such as stimulating growth hormone release, modulating glucose metabolism, enhancing learning and memory, and increasing cardiac output [3]. Besides these effects on food intake and energy homeostasis, ghrelin can be also synthesized in several

other tissues such as human ovary and testis [4], suggesting that ghrelin regulates several aspects of reproductive physiology and pathology. Two growth hormone secretagogue receptor (GHS-R) subtypes, generated by alternative splicing of a single gene, have been identified so far. The full-length GHS-R1a is the fully functional receptor, while the truncated GHS-R1b lacks transmembrane domains 6 and 7, and thus has low capacity of high-affinity ligand-binding and signal transduction [5, 6]. In the male reproductive system, the expression of GHS-R1a and its ligand are fairly consistent throughout the postnatal development and are regulated by hormonal signals, in particular stimulated by ghrelin [4, 7], which is present in the testis [2]. This suggests that, by regulating GHS-R, ghrelin plays a role in fertilization, such as sperm transport through the female genital tract. In addition to its steroidogenic effects, ghrelin might directly regulate seminiferous tubule functions, as expression of GHS-R1a is demonstrated in the tubular compartment of the testis [4, 8]. Moreover, ghrelin is also reported to induce a significant inhibition of cAMP-stimulated testosterone secretion and sperm motility *in vitro* [9]. However, to date, the detailed mechanisms underlying these effects induced by ghrelin remain obstacle.

T-type Ca^{2+} channels play an essential role in the initiation of sperm-oocyte interaction, and are required for acrosome reaction (AR) and sperm motility [10]. Previous studies showed that AR was inhibited by Ca^{2+} channel blockers or in Ca^{2+} -free medium [10, 11]. Furthermore, it was reported that the intake of Ca^{2+} channel blockers could elicit contraceptive effects in men [12]. These results indicated that the inhibition of Ca^{2+} currents could be responsible for the infertility side-effects of these drugs. However, due to the small size, complex geometry, highly differentiated and motile nature of sperm, it is difficult to study ion channels directly in mature sperms by the patch-clamp electrophysiological method. T-type Ca^{2+} channels are expressed during the meiotic and post-meiotic stages of spermatogenesis and are retained in mature sperms. They play an important role in the regulation of $[\text{Ca}^{2+}]_i$ during the AR and sperm motility [11-14]. Therefore, the spermatogenic cell, a developmental precursor of mature sperm, is usually used as a model system to study the effects of agents on the channel activities in mature sperms [13-15].

In the present study, we investigated the effect of ghrelin on T-type Ca^{2+} channel currents (T-currents) in mouse spermatogenic cells. Based on pharmacological manipulation of ghrelin-induced T-current inhibition, we

reported that ghrelin inhibited T-currents via a pertussis toxin-sensitive novel protein kinase C pathway and might contribute to the male reproductive functions such as sperm AR.

Materials and Methods

All animal care and handling conformed to the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health, and the study was approved by the local institutional ethical committee.

Reagents and solutions

All drugs were obtained from Sigma (USA), unless otherwise indicated. Ghrelin, GDP- β -S, pertussis toxin (PTX), cholera toxin (CTX), PKI 6-22, chelerythrine chloride and Ro 31-8220 were prepared in distilled deionized water. Stock solutions of U73122, U73343, forskolin, GF109203X and D-Lys3-GHRP-6 were prepared in dimethyl sulfoxide (DMSO). The concentration of DMSO in the bath solution is expected to be less than 0.01%, and had no functional effects on T-type Ca^{2+} channels (data not shown). The composition of the external solution for T-type Ca^{2+} channel current recording was composed of (in mM): 5 BaCl_2 , 130 NaCl , 3 KCl , 2 MgCl_2 , 1 NaHCO_3 , 0.5 NaH_2PO_4 , 5 HEPES, 10 glucose, pH adjusted with NaOH to 7.3. The pipette solution contained (in mM): 100 CsCl , 10 CsF , 5 EGTA, 5 HEPES, 4 Mg-ATP , 4 phosphocreatine, pH adjusted with CsOH to 7.3.

Reverse transcription-PCR (RT-PCR)

Total RNA was extracted from mouse testis described by our previous reports [16]. cDNA was reverse transcribed from total RNA using SuperScriptTMII (Invitrogen). The sequences of the primers were as following, GHS-R1a sense: 5'-CCG CCT CTG GCA GTA TCG-3'; anti-sense: 5'-GCT GAC AAA CTG GAA GAG TTT GC-3' and β -actin sense: 5'-CAT CTT GGC CTC A CT GTC CAC-3', anti-sense: 5'-GGG CCG GAC TCA TCG TAC T-3'. The cycling parameters for PCR amplification included 1 cycle at 95°C for 2 min, followed by 35 cycles at 94°C for 30 sec, 65°C for 20 sec and 72°C for 1 min, and a final extension at 72°C for 10 min. PCR was performed at least twice for the same samples to confirm reproducibility.

Western blot

Western blot analysis was performed following the procedure described by our previous reports [16]. For antibody detection, after incubated with 5% non-fat milk in TBST for 1 h at room temperature, membranes were then incubated with diluted primary antibody goat anti-mouse GHS-R1a (Santa Cruz, 1:500) and incubated at 4 °C overnight. After 5 washes with TBST, membranes were incubated for 2 h with diluted 5000-fold donkey anti-goat secondary antibody (Sigma, USA). After 5 washes, the specific binding of the primary antibody was detected with SuperSignal Ultra chemiluminescent substrate (Pierce).

Fig. 1. Expression of GHS-R1a in mouse testis. A, expression of GHS-R1a mRNA was determined by reverse transcription PCR using total RNA from mouse testis (291 bp). Total RNA from mouse was used as positive control. RNA from testis that was not reverse-transcribed served as negative control (-RT). Comparable loading was verified by amplification of β -actin. PCR products were analyzed on 1% TAE agarose gel. B, expression of GHS-R1a protein in mouse testis was determined by western blot (~ 45 kDa). Mouse hippocampus was used as a positive control. Comparable loading was verified by β -actin. C, Immunohistochemical staining of GHS-R1a on formalin fixed paraffin embedded mouse testis sections. Goat IgG was served as isotype control. Scar bar, 50 μ m.

Immunohistochemical staining

Immunohistochemistry was performed as previously described [17]. Briefly, sections were incubated in 1% hydrogen peroxide, washed in PBS and non-specific protein binding was blocked with goat serum. Sections were then incubated overnight in a humid chamber at 4°C with polyclonal goat anti-mouse GHS-R1a antibody (Santa Cruz, 1:500). Following three PBS washes, sections were incubated with HRP conjugated rabbit anti-goat secondary antibody. Positive cells were visualized brown with diaminobenzidine and mounted for bright field microscopy.

Spermatogenic cell preparation and electrophysiology

Spermatogenic cells were obtained following the procedure described by our previous reports [18]. Recordings were made using standard whole-cell techniques at room temperature. A small aliquot (0.1 ml) of isolated cells was placed in a 0.5 ml chamber mounted on an inverted microscope (Ti-S, Nikon). Cells were allowed to adhere to the coverslip and superfused at room temperature (19-23°C). The currents were recorded and dealt with by an amplifier (Axon 700B, Molecular Device), Digidata 1440A, and p-Clamp software 9.2 (Molecular Device, USA). Borosilicate glass electrodes were pulled using a level puller (Sutter Instruments, Model P-97) and had a resistance of 2 to 5 M Ω when filled with the electrode internal solution. After gigaseal and membrane rupture, the electrode resistance in series to the cell membrane was compensated at least 75%, and the T-currents were recorded in a voltage-clamp mode. Cell capacitance was measured by integrating the area of the capacitive transient. T-currents were elicited by a 100 ms-long depolarizing step pulse from the holding potential of -90 mV to -20 mV. Pulses from -80 mV to +40 mV in 10 mV increments were used to elicit the membrane

currents for obtaining of I-V curve. The steady-state activation curve of T-current was well fitted by the Boltzmann equation: $G/G_{\max} = 1/\{1+\text{EXP}[(V-V_{0.5})/\kappa]\}$, where $G_{\max}$ is the maximal conductance. $V_{0.5}$ is the half activation voltage, and κ is the slope factor. Steady-state inactivation curves were obtained by applying test pulses for 50 ms from -90 mV to -20 mV, 5 ms after 2-s conditioning pulses ranging from -100 mV to -10 mV and were fitted with the Boltzmann equation: $I/I_{\max} = 1/\{1+\text{EXP}[(V-V_{0.5})/\kappa]\}$, where $V_{0.5}$ and κ are the half-maximum inactivation potential and the slope factor, respectively.

Assay for acrosome reaction

Acrosome reaction was performed following the procedure described by our previous reports [18]. Sperm acrosomal status was examined under a fluorescence microscope in which an Hg excitation beam passed through a 405-nm filter and fluorescence emission was monitored using a DM 455 dichroic mirror (Olympus IX70, Japan). To calculate the percentage of AR, A total of 100 spermatozoa per experimental condition were assayed to assess the different chlortetracycline staining patterns.

Data analysis

Data were expressed as means $\pm$ S.E.M.. The statistical software used was Prism 5.0. Student's *t*-tests or one-way analysis of variance (ANOVA) with *post hoc* Bonferroni was used to compare the different values, and were considered significant at $P<0.05$, $P<0.01$, or $P<0.001$. Concentration-response curves were fitted by sigmoidal Hill equation $I/I_{\text{control}} = 1/(1+10^{(\log \text{IC}_{50}-X)n_H})$, where X is the decadic logarithm of the concentration used, IC_{50} is the concentration at which the half-maximum effect occurs, and n_H is the Hill coefficient.

Fig. 2. Inhibitory effect of ghrelin on T-currents in mouse spermatogenic cells. A, Original records of the currents evoked by a series of depolarizing pulses from a holding potential of -90 mV to test potentials between -80 and +40 mV, in 10 mV increment *per* step. B, pooled data of effects of Bay K8644 (1 μ M), Ni²⁺ (200 μ M), and mibefradil (100 μ M) on T-currents. C, time course of changes in amplitude of T-currents under control conditions (a), during exposure to 0.1 μ M ghrelin (b), and wash out (c). D, exemplary traces of T-currents recorded under conditions indicated in panel B. The dotted line above current traces represents the zero current level. E, dose-response relationship for the inhibitory effects of ghrelin on T-currents. The line represents the best fit of the data points to the sigmoidal Hill equation (see “Methods and Materials”). The number of cells tested at each concentration of ghrelin is indicated in brackets. F, Effects of ghrelin at 0.1 μ M on current-voltage (I-V) curve. *P<0.05 vs. control, **P<0.01 vs. control, ***P<0.001 vs. control.

Fig. 3. Effects of ghrelin on T-type Ca²⁺ channel electrophysiological properties. A, The steady-state activation of T-type Ca²⁺ channels is not altered by ghrelin application (n=7). Tail currents were elicited by repolarization to -90 mV after 50 ms test pulses from -80 to 0 mV in increments of 10 mV. B, Steady-state inactivation of ghrelin on T-type Ca²⁺ channels. T-currents evoked by 50 ms test pulse to -20mV after the 2 s conditioning pulses ranging from -100 to 0 mV with 10 mV increment *per* step. Insets show the exemplary traces respectively in panel A and panel B.

Results

Expression of GHS-R1a in mouse testis

To determine whether functional GHS type 1a receptors (GHS-R1a) were endogenously expressed in

mouse testis, we checked mRNA and protein expressions by reverse transcription -PCR and western blot, respectively. Mouse hippocampus, which is known to express GHS-R1a [3], served as positive controls. Indeed, we found both mRNA (Fig. 1A) and protein (Fig. 1B) of

Fig. 4. Ghrelin inhibited T-currents via GHS-R1a. A, time course of changes in amplitude of T-currents induced by ghrelin (0.1 μ M) in the presence of D-Lys3-GHRP-6 (1 μ M). B, exemplary current traces indicated in panel A. C, pooled data showed the effects of ghrelin (0.1 μ M) on T-currents in the presence of D-Lys3-GHRP-6 (1 μ M). *** P <0.001 vs. control.

GHS-R1a were expressed in mouse testis. The expression was further confirmed by immunohistochemistry staining of GHS-R1a on mouse testis sections. It clearly showed that GHS-R1a was expressed in both spermatogenic cells and mature spermatozoa (Fig. 1C). Isotype control (goat IgG) was used to confirm the specificity of staining.

Ghrelin inhibited T-type Ca^{2+} channel currents in mouse spermatogenic cells

T-type Ca^{2+} channel currents (T-currents) in mouse spermatogenic cells were characterized first. As shown in Fig. 2A, a series of evoked current traces were recorded when a spermatogenic cell was given designed depolarizing test pulses. There were few steady state components in the current (Fig. 2A). In addition, the current was not affected by 1 μ M Bay K8644, a specific L-type Ca^{2+} channel activator ($n=5$, Fig. 2B). After perfusion with 200 μ M Ni^{2+} -containing solution, the amplitude of basal currents was drastically decreased ($n=6$, Fig. 2B). Similar results were obtained by application of mibefradil (100 μ M), another T-type Ca^{2+} channel blocker ($n=6$, Fig. 2B). The low voltage-activated, fast inactivating, steady state component-free and Ni^{2+} -sensitive current showed typical properties of the T-type Ca^{2+} currents, which is also in accordance with previous reports that only T-type Ca^{2+} channels were detected in mouse spermatogenic cells [10, 16–18].

Bath application of 0.1 μ M ghrelin inhibited the amplitude of basal T-currents in spermatogenic cells by $31.71 \pm 2.53\%$ ($n=7$, $P<0.01$, Fig. 2C). Upon washout

of ghrelin, the amplitude of T-currents partially returned within 3 min (Fig. 2C and D), which indicated that the effect of ghrelin on T-currents was not due to the rundown. Moreover, ghrelin inhibited T-currents in a dose-dependent manner (Fig. 2E). The relationship between the concentration of ghrelin used and the degree of inhibition observed was described by a logistic equation where the concentration of ghrelin producing half-maximal inhibition (IC_{50}) was 35.7 nM, the apparent Hill coefficient was 0.92, and the maximal inhibitory effect was $38.3 \pm 2.6\%$ ($n=7$, $P<0.01$, Fig. 2E). Furthermore, the level of T-currents inhibition produced by 0.1 μ M ghrelin was independent of the voltage (Fig. 2F). Population data showed that 0.1 μ M ghrelin up-shifted the I-V curve at each voltage, and at -20 mV the current density declined from 7.6 ± 0.3 pA/pF to 5.3 ± 0.8 pA/pF ($n=7$, Fig. 2F).

Ghrelin shifted the inactivation curve of T-type Ca^{2+} channels

We further investigated whether the biophysical properties of T-type Ca^{2+} channels were affected by ghrelin. Steady-state activation and inactivation potentials of T-type Ca^{2+} channels were examined. We observed a slight but statistically insignificant shift of 2.9 mV in the hyperpolarized direction of the activation potential ($V_{1/2}$ from -41.6 ± 0.5 to -44.1 ± 0.9 mV, and k value from 5.9 ± 0.5 to 6.3 ± 0.8 , $n=7$, $P>0.05$) (Fig. 3A). In contrast, ghrelin significantly shifted the steady-state inactivation potentials of T-type Ca^{2+} channels in the hyperpolarizing direction by ~ 11.2 mV ($V_{1/2}$

Fig. 5. Ghrelin-induced T-current inhibition was pertussis toxin-sensitive. A-C, exemplary traces and pooled data showed the effects ghrelin (0.1 μ M) on T-currents in the presence of GDP- β -S (1 mM, A), CTX (0.5 μ g/ml, B), and PTX (0.2 μ g/ml, C), respectively. *** P <0.001 vs. control. D, exemplary traces and pooled data showed the effects ghrelin (0.1 μ M) on T-currents in the presence of QEHA. E, Ghrelin (0.1 μ M) induced similar T-current inhibition in the presence or absence of prepulse depolarization. T-currents evoked by depolarizing steps to -20 mV, from a holding potential of -90 mV, preceded by a prepulse to +80 mV.

from -48.5 ± 1.7 to -58.6 ± 0.6 mV, and k value from 5.9 ± 0.9 to 6.6 ± 0.5 , $n=7$, $P<0.05$) (Fig. 3B). These results together suggested that the reduction in T-currents observed upon application of ghrelin in mouse spermatogenic cells was due to an increased proportion of channels remaining in the inactivated state.

Ghrelin inhibited T-currents via GSH-R1a

GHS-R1a has been identified as the endogenously functional receptors for ghrelin *in vivo* [7]. To determine

whether ghrelin exerts its effects via its GHS-R1a, we examined its effects on T-currents in the presence of ghrelin receptor antagonists (Fig. 4A). Our results showed that pretreatment of the cells with D-Lys3-GHRP-6 (1 μ M), a selective GHS-R1a antagonist, abolished ghrelin-induced T-current responses in mouse spermatogenic cells (inhibition% = 1.9 ± 2.4 , $n=5$, $P<0.001$) (Fig. 4B and C), which suggested that inhibition of T-currents by ghrelin was dependent of its endogenous GHS-R1a.

Fig. 6. Ghrelin inhibited T-currents through novel PKC pathway.

A-B, exemplary traces and pooled data showed the inhibition of T-currents induced by ghrelin (0.1 μ M, A) or forskolin (20 μ M, B) in the presence of PKI 6-22 (1 μ M). C-H, exemplary traces and pooled data showed the inhibition of T-currents induced by ghrelin (0.1 μ M) in the presence of GF109203X (1 μ M, C), chelerythrine chloride (1 μ M, D), Ro 31-8220 (1 μ M, E), U73122 (3 μ M, F), *** P <0.001 vs. control.

GHS-R1a-mediated T-current inhibition was pertussis toxin-sensitive

Since GHS-R1a belongs to a family of G-protein coupled receptors (GPCRs) [4, 5], we further investigated whether heterotrimeric G-proteins were involved in ghrelin-mediated inhibition of T-currents, by dialyzing the cells with guanosine-5'-O-(2-thiodiphosphate) (GDP- β -S, 1 mM), a non-hydrolysable GDP analog. Our results showed that GDP- β -S completely abolished the inhibition of T-currents induced by 0.1 μ M ghrelin (inhibition%=1.3 $\pm$ 0.9, n=6, P <0.001, Fig. 5A), indicating that G-protein activation was required for ghrelin

action. We further determined which isoform of G α was involved in this inhibition. We examined the inhibitory effects of ghrelin on T-currents in the presence of cholera toxin (CTX, 0.5 μ g/ml), which inactivates G α_s by ADP-ribosylation. After pretreatment of cells with CTX, ghrelin still robustly inhibited T-currents (inhibition%=30.9 $\pm$ 2.7, n=7, Fig. 5B). However, pretreatment of the cells with pertussis toxin (PTX, 0.2 μ g/ml), which catalyzes the ADP-ribosylation of G $\alpha_{i/o}$, abolished the inhibitory effect of ghrelin (inhibition%=3.1 $\pm$ 0.9, n=7, Fig. 5C). The inhibition of T-currents induced by ghrelin was sensitive to PTX,

but not CTX, indicating that $G_{i/o}\alpha$, but not $G_s\alpha$, was involved.

We further investigated the possible involvement of the G-protein $\beta\gamma$ subunits ($G_{\beta\gamma}$) in GHS-R1a-mediated T-current inhibition. In heterologous expression systems, it has been shown that T-type Ca^{2+} channels can be blocked by a direct binding of $G_{\beta\gamma}$ on the T-type Ca^{2+} channel subunits [19]. To determine whether GHS-R1a-mediated T-current inhibition was via $G_{\beta\gamma}$ direct binding, we disrupted the interactions between $G_{\beta\gamma}$ and T-type Ca^{2+} channel subunit in two ways. First, a synthetic peptide, QEHA (200 μ M) (encoding residues 956 to 982 of ACII), which competitively binds $G_{\beta\gamma}$ and blocks $G_{\beta\gamma}$ -mediated signaling [20], was introduced into the recording pipette. Our results showed that the inhibitory effect of ghrelin was not significantly altered by intracellular applying QEHA (inhibition% = 30.7 ± 1.5 , $n=7$, Fig. 5D). Second, we utilized a depolarizing prepulse to disrupt any potential $G_{\beta\gamma}$ and the T-type Ca^{2+} channel interaction. In mouse spermatogenic cells, no such voltage dependent facilitation was observed in the absence or presence of ghrelin (Fig. 5E). Thus the ratio, R , between amplitudes of T-currents evoked with and without depolarizing prepulse was measured in the absence and presence of ghrelin. We obtained values of R that were not significantly different ($R = 0.76 \pm 0.31\%$, $n=6$, in the absence of ghrelin; $R = 0.73 \pm 0.27\%$, $n=6$, in the presence of ghrelin). Furthermore, ghrelin at 0.1 μ M induced similar T-current inhibition in the presence (inhibition% = 32.3 ± 2.9 with prepulse, $n=7$, Fig. 5E) or absence of prepulse depolarization. These results strongly suggested the ghrelin-induced T-current inhibition was not through the direct interaction of $G_{\beta\gamma}$ subunits with T-type Ca^{2+} channels in mouse spermatogenic cells.

Involvement of novel protein kinase C isoform in ghrelin-induced T-current inhibition

Since the activation of GHS-R1a by ghrelin inhibited spermatogenic cell T-currents via an indirect action of a $G_{i/o}$ -protein, we searched for the involvement of intermediate intracellular factors. To determine whether protein kinase A (PKA) was involved in ghrelin-induced T-current inhibition, we dialyzed the cells with a pipette solution containing the PKA inhibitor, PKI 6-22. Our results showed that intracellular application of PKI 6-22 (1 μ M) had no effects on ghrelin-induced T-current inhibition in spermatogenic cells (inhibition% = 32.2 ± 1.6 , $n=7$, Fig. 6A). The PKA inhibitor, PKI 6-22, used here was effective since pretreatment of spermatogenic

Fig. 7. Effects of ghrelin on acrosome reaction (AR) evoked by progesterone. A, Inhibition of ghrelin on AR evoked by progesterone. B, D-Lys (1 μ M) or mibefradil (100 μ M) abolished the AR inhibition induced by ghrelin. ** $P < 0.01$ vs. control, *** $P < 0.001$ vs. control.

cells with 1 μ M PKI 6-22 abolished forskolin (20 μ M)-induced T-current inhibition as reported in previous studies [20]. The inhibition of T-currents were reduced from $28.6 \pm 2.1\%$ ($n=7$) to $3.6 \pm 2.1\%$ ($n=7$) by PKI 6-22 (Fig. 6B). These results strongly suggested that ghrelin-induced T-current inhibition was PKA-independent. Since it was reported that T-type Ca^{2+} channels could be regulated by the PKC pathway [20], we investigated whether the inhibitory effects of ghrelin were mediated by a PKC-dependent pathway. The pretreatment of spermatogenic cell with

Fig. 8. Model for GHS-R1a-mediated inhibition of T-type Ca^{2+} channels in mouse spermatogenic cells. Ghrelin acts through GHS-R1a to activate $\text{G}_{i/o}$ α -proteins and release $\text{G}_{\beta\gamma}$ subunits. Activation of PLC by $\text{G}_{i/o}$ -protein stimulates novel PKC (nPKC) epsilon isoform, which in turn blocks the T-type Ca^{2+} channels. Neither PKA nor direct interaction between $\text{G}_{\beta\gamma}$ and T-type Ca^{2+} channels is necessary for GHS-R1a-mediated inhibition of T-currents. Whether PKC ϵ directly phosphorylates T-channels or acts on an intermediate protein needs to be further determined.

GF109203X (1 μM), a classical and novel PKC antagonist, abolished ghrelin-induced T-current responses (inhibition% = 2.5 ± 0.3 , $n=7$, Fig. 6C). Similar results were obtained with another classical and novel PKC antagonist chelerythrine chloride (inhibition% = 1.3 ± 1.7 , $n=7$, Fig. 6D). However, pre-incubation of the cells with the classical PKC antagonist Ro 31-8220 (200 nM) elicited no such effects (inhibition% = 34.5 ± 2.6 , $n=7$, Fig. 6E), suggesting a novel PKC (nPKC) pathway was involved. In rat Purkinje neurons, $\text{G}_{i/o}$ α protein activation can lead to nPKC isoforms activation via phospholipase C (PLC) [16]. Indeed, we found that the ghrelin-induced inhibitory effect was abolished by the PLC inhibitor U73122 (3 μM) (inhibition% = 3.1 ± 1.9 , $n=7$, Fig. 6F). As a control, a structurally related but inactive analogue U73343 (3 μM) had no such effect (Data not shown). These results indicated that ghrelin inhibited T-type Ca^{2+} channels via a PLC-dependent novel PKC pathway.

Ghrelin inhibited sperm acrosome reaction in mice

To further characterize the functions of ghrelin in male reproduction, we studied the effects of ghrelin on sperm acrosome reaction (AR) in mice. Progesterone (15 μM), the physiological agonist of AR, was used to evoke the AR. Spontaneous AR occurred in $19.7 \pm 2.7\%$ of incubated cells whereas $62.8 \pm 2.7\%$ AR was observed

in progesterone-induced cells (Fig. 7A). The progesterone-evoked AR was inhibited by ghrelin concentrations ranging from 0.01 μM to 1 μM (Fig. 7A). Pretreatment of cells with a GHS-R1a antagonist, D-Lys3-GHRP-6 (1 μM), completely blocked the ghrelin-induced inhibition of AR (Fig. 7B), which suggested the ghrelin-induced inhibition of AR was through GHS-R1a. Mibefradil, an antagonist of T-type Ca^{2+} channels, was reported to inhibit T-type Ca^{2+} channels in spermatogenic cells and reduced sperm AR in mice [21]. In the present study, Mibefradil at 100 μM significantly inhibited the progesterone-evoked AR by $30.6 \pm 4.3\%$ ($P < 0.001$). To test whether ghrelin inhibits AR via T-type Ca^{2+} channels, we investigated whether mibefradil would occlude ghrelin-mediated AR inhibition. Indeed, application of 100 μM mibefradil mimicked ghrelin's inhibition of AR (Fig. 7B). Notably, application of ghrelin during the maximum mibefradil-induced response failed to produce any further inhibition (Fig. 7B). These results suggested that ghrelin inhibited sperm AR through its GHS-R1a and T-type Ca^{2+} channels.

Discussion

In the present study, we first demonstrate that ghrelin acts through GHS-R1a and plays a novel role in modulating T-type Ca^{2+} channels in mouse spermatogenic cells. Based on pharmacological manipulation of ghrelin-induced T-current inhibition, we propose that this response is coupled to a pertussis toxin (PTX)-sensitive and phospholipase C (PLC)-dependent novel protein kinase C (PKC) pathway (Fig. 8) and might contribute to its male reproductive functions such as acrosome reactions.

Indirect G-protein mediated inhibition of T-currents by GHS-R1a

GHS-R1a belongs to a family of functional G-protein coupled receptors (GPCR). Previous results suggest that different G-proteins are activated with respect to the GPCR: one is PTX-insensitive, while the other is PTX-sensitive [22]. In our present study, the inhibitory effects of ghrelin on T-currents in mouse spermatogenic cells were PTX-sensitive because: 1) the effect was blocked by the nonselective G-protein inhibitor GDP- β -S; 2) this inhibition was abolished by PTX, but not cholera toxin (CTX). Our electrophysiological data are supported by previous reports that GHS-R1a receptor-mediated cAMP/PKA activation was

prevented by pretreatment with PTX [23], suggesting coupling to $G_{i/o}$ proteins. It is reported that $G_{\beta\gamma}$ could interact directly with T-type Ca^{2+} channels, as was observed for recombinant Cav3 channels expressed in human embryonic kidney (HEK) 293 cells and for T-currents recorded in hippocampal neurons [24]. However, such a $G_{\beta\gamma}$ mechanism is unlikely to mediate GHS-R1a inhibition of T-currents in mouse spermatogenic cells because ghrelin-induced inhibition was neither abrogated by a depolarizing prepulse nor by the intracellular application of QEHA, a $G_{\beta\gamma}$ antagonist. Our data suggest that ghrelin-mediated GHS-R1a activation does not result in direct binding between $G_{\beta\gamma}$ and T-type Ca^{2+} channels. In addition, the inhibitory mechanism of T-type Ca^{2+} channels mediated by GHS-R1a is also distinctly different from the activation of the GPCRs including dopamine receptors in hippocampal neurons [24]. Our data suggest that the ghrelin-mediated inhibition of T-current is through the stabilization of the channels in their inactivated state, whereas activation of dopamine 1 (D1) receptors reduces the open probability of the channels, and the voltage dependent steady-state inactivation of the channels remains unchanged [24].

Novel PKC is an intracellular mediator of ghrelin's action downstream of GHS-R1a

There are several examples of T-current modulation by PKA activation. In retinal horizontal cells, T-current inhibition by dopamine is prevented by application of PKA inhibitors [25]. Similarly, T-current inhibition by adrenaline in newt olfactory receptor cells is mimicked by intracellular application of the catalytic subunit of PKA [26]. By contrast, the inhibition of recombinant T-type Ca^{2+} channels by D1 receptor persists in the presence of a PKA peptide inhibitor, and the catalytic subunit of PKA added into the patch-pipette produces no effect on recombinant T-type Ca^{2+} channels [27]. In addition, PKA-induced nickel-sensitive T-current increase is also described in frog atrial myocytes and in recombinant HEK293 cells [20]. In the present study, we find that the inhibitory effect of ghrelin on T-currents is independent of PKA, which suggests that some other mechanisms, but not cAMP/PKA pathway, are involved in the inhibition of T-currents induced by ghrelin. Our results show that pretreatment of cells with classic and novel PKC (nPKC) inhibitor, but not the classic PKC inhibitor, could completely abolish the inhibition of ghrelin on T-currents in mouse spermatogenic cells, which suggests that an nPKC pathway is involved in GHS-R1a-mediated T-current

inhibition. Our present results are supported by previous studies that activation of PKC by phorbol 12-myristate 13-acetate (PMA) inhibits T-currents while an inactive analogue has no effect in canine Purkinje and ventricular cells [28]. However, PKC-induced nickel-sensitive T-current increase is also described in chick ventricular myocytes [20]. For example, endothelin-1 induces a T-current increase that is prevented by PKC inhibitors H7 and staurosporine while it is mimicked by PMA and phorbol-12,13-dibutyrate (Pdbu). Importantly, PMA effects are abolished by pre-incubation with different PKC inhibitors. Interestingly, these results are not reproduced in mammalian cells [29] and therefore may depend on different species. Together, these results suggest that the PKC regulatory effects on T-currents can be variable in different tissues expressing T-type Ca^{2+} channels that are functionally coupled to different G-protein coupled receptors.

Functional perspectives of T-channel inhibition induced by GHS-R1a

Acrosome reaction (AR) is highly associated with T-type Ca^{2+} channels in spermatozoa [10]. In mammalian, AR is a key step in sperm-oocyte membrane fusion, which could be inhibited by calcium channel blockers [11]. In the present study, we demonstrate that activation of GHS-R1a inhibits progesterone-induced AR in mouse sperm via inhibiting T-type Ca^{2+} channels. Our present results are consistent with the previous reports implicating role of T-type Ca^{2+} channels in sperm functions. It has been reported that sperm-specific CatSper1 and CatSper2 proteins are critical to sperm-hyperactivated motility and male fertility [30]. Recent reports show that CatSper1 and CatSper2 can associate with and modulate the function of the T-type Ca^{2+} channels, which might be important in the regulation of sperm functions including AR [31]. Also, the inhibition of the progesterone-initiated AR by mibefradil suggests the involvement of T-type Ca^{2+} channels [32]. In addition, it was shown that a Ca_v3 expressed in the *Strongylocentrotus purpuratus* and participated in the regulation of sperm AR [33]. Although further investigations are needed, all of these reports suggest that T-type Ca^{2+} channels participate in some sperm functions like motility, capacitation, and AR. Together these results demonstrate that activation of GHS-R1a by ghrelin plays important roles in the male reproductive functions by inhibiting T-type Ca^{2+} channels.

In conclusion, this study presents a novel action of ghrelin on T-currents in mouse spermatogenic cells. A

PTX-sensitive and PLC-dependent novel PKC pathway downstream of GHS-R1a mediates inhibitory effects on T-currents and AR. In further studies, transfected HEK293 cells will be used to distinguish the inhibitory effects of ghrelin on T-type Ca^{2+} channels through Cav3.1 or Cav3.2 channels, either of which is the main functional Ca^{2+} channel in mouse spermatogenic cells [34, 35].

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References

- Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K: Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 1999;402:656-660.
- Lorenzi T, Meli R, Marziani D, Morroni M, Baragli A, Castellucci M, Gualillo O, Muccioli G: Ghrelin: a metabolic signal affecting the reproductive system. *Cytokine Growth Factor Rev* 2009;20:137-152.
- Korbonits M, Goldstone AP, Gueorguiev M, Grossman AB: Ghrelin-a hormone with multiple functions. *Front Neuroendocrinol* 2004;25:27-68.
- Tena-Sempere M: Ghrelin as a pleiotrophic modulator of gonadal function and reproduction. *Nat Clin Pract Endocrinol Metab* 2008;4:666-674.
- McKee KK, Palyha OC, Feighner SD, Hreniuk DL, Tan CP, Phillips MS, Smith RG, van der Ploeg LH, Howard AD: Molecular analysis of rat pituitary and hypothalamic growth hormone secretagogue receptors. *Mol Endocrinol* 1997;11:415-423.
- Smith RG, van der Ploeg LHT, Howard AD, Feighner SD, Cheng K, Hickey GJ, Wyvratt MJ, Fisher MH, Nargund RP, Patchett AA: Peptidomimetic regulation of growth hormone secretion. *Endocr Rev* 1997;18:621-645.
- Gaytan F, Barreiro ML, Caminos JE, Chopin LK, Herington AC, Morales C, Pinilla L, Paniagua R, Nistal M, Casanueva FF, Aguilar E, Dieguez C, Tena-Sempere M: Expression of ghrelin and its functional receptor, the type 1a growth hormone secretagogue receptor, in normal human testis and testicular tumors. *J Clin Endocrinol Metab* 2004;89:400-409.
- Tena-Sempere M: Exploring the role of ghrelin as novel regulator of gonadal function. *Growth Horm IGF Res* 2005;15:83-88.
- Tena-Sempere M, Barreiro ML, Gonzalez LC, Gaytan F, Zhang FP, Caminos JE, Pinilla L, Casanueva FF, Dieguez C, Aguilar E: Novel expression and functional role of ghrelin in rat testis. *Endocrinology* 2002;143:717-725.
- Darszon A, Labarca P, Nishigaki T, Espinosa F: Ion channels in sperm physiology. *Physiol Rev* 1999;79:481-510.
- Espinosa F, Lopez-Gonzalez I, Munoz-Garay C, Felix R, De La Vega-Beltran JL, Kopf GS, Visconti PE, Darszon A: Dual regulation of the T-type Ca^{2+} current by serum albumin and beta-estradiol in mammalian spermatogenic cells. *FEBS Lett* 2000;475:251-256.
- Goodwin LO, Leeds NB, Hurley I, Mandel FS, Pergolizzi RG, Benoff S: Isolation and characterization of the primary structure of testis-specific L-type calcium channel: implications for contraception. *Mol Hum Reprod* 1997;3:255-268.
- Lievano A, Santi CM, Serrano CJ, Trevino CL, Bellve AR, Hernandez-Cruz A, Darszon A: T-type Ca^{2+} channels and $\alpha\text{h}1\text{E}$ expression in spermatogenic cells, and their possible relevance to the sperm acrosome reaction. *FEBS Lett* 1996;388:150-154.
- Arnoult C, Villaz M, Florman HM: Pharmacological properties of the T-type Ca^{2+} current of mouse spermatogenic cells. *Mol Pharmacol* 1998;53:1104-1111.
- Espinosa F, Lopez-Gonzalez I, Serrano CJ, Gasque G, De La Vega-Beltran JL, Trevino CL, Darszon A: Anion channel blockers differentially affect T-type Ca^{2+} currents of mouse spermatogenic cells, $\alpha\text{h}1\text{E}$ currents expressed in *Xenopus* oocytes and the sperm acrosome reaction. *Dev Genet* 1999;25:103-114.
- Zhang Y, Jiang D, Zhang J, Wang F, Jiang X, Tao J: Activation of neuromedin U type 1 receptor inhibits L-type Ca^{2+} channel currents via phosphatidylinositol 3-kinase-dependent protein kinase C epsilon pathway in mouse hippocampal neurons. *Cell Signal* 2010;22:1660-1668.
- Tao J, Lin M, Sha J, Tan G, Soong TW, Li S: Separate locations of urocortin and its receptors in mouse testis: function in male reproduction and the relevant mechanisms. *Cell Physiol Biochem* 2007;19:303-312.
- Tao J, Zhang Y, Li S, Sun W, Soong TW: Tyrosine kinase-independent inhibition by genistein on spermatogenic T-type calcium channels attenuates mouse sperm motility and acrosome reaction. *Cell Calcium* 2009;45:133-143.
- Tao J, Hildebrand ME, Liao P, Liang MC, Tan G, Li S, Snutch TP, Soong TW: Activation of corticotropin-releasing factor receptor 1 selectively inhibits Cav3.2 T-type calcium channels. *Mol Pharmacol* 2008;73:1596-1609.
- Chemin J, Traboulsie A, Lory P: Molecular pathways underlying the modulation of T-type calcium channels by neurotransmitters and hormones. *Cell Calcium* 2006;40:121-134.
- Trevino CL, Felix R, Castellano LE, Gutiérrez C, Rodríguez D, Pacheco J, López-González I, Gomora JC, Tsutsumi V, Hernández-Cruz A, Fiordelisio T, Scaling AL, Darszon A: Expression and differential cell distribution of low-threshold Ca^{2+} channels in mammalian male germ cells and sperm. *FEBS Lett* 2004;563:87-92.
- Couty JP, Geras-Raaka E, Weksler BB, Gershengorn MC: Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor signals through multiple pathways in endothelial cells. *J Biol Chem* 2001;276:33805-33811.

- 23 Rossi F, Castelli A, Bianco MJ, Bertone C, Brama M, Santiemma V: Ghrelin inhibits contraction and proliferation of human aortic smooth muscle cells by cAMP/PKA pathway activation. *Atherosclerosis*. 2009;203:97-104.
- 24 Wolfe JT, Wang H, Howard J, Garrison JC, Barrett PQ: T-type calcium channel regulation by specific G-protein betagamma subunits. *Nature* 2003;424:209-213.
- 25 Pfeiffer-Linn CL, Lasater EM: Multiple second-messenger system modulation of voltage-activated calcium currents in teleost retinal horizontal cells. *J Neurophysiol* 1998;80:377-388.
- 26 Michel WC, Ache BW: Cyclic nucleotides mediate an odor-evoked potassium conductance in lobster olfactory receptor cells. *J Neurosci* 1992;12:3979-3984.
- 27 Wetzel CH, Spehr M, Hatt H: Phosphorylation of voltage-gated ion channels in rat olfactory receptor neurons. *Eur J Neurosci* 2001;14:1056-1064.
- 28 Tseng GN, Boyden PA: Different effects of intracellular Ca and protein kinase C on cardiac T and L Ca currents. *Am J Physiol* 1991;261:H364-379.
- 29 Park JY, Jeong SW, Perez-Reyes E, Lee JH: Modulation of Cav3.2 T-type Ca²⁺ channels by protein kinase C. *FEBS Lett* 2003;547:37-42.
- 30 Ren D, Navarro B, Perez G, Jackson AC, Hsu S, Shi Q, Tilly JL, Clapham DE: A sperm ion channel required for sperm motility and male fertility. *Nature* 2001;413:603-609.
- 31 Zhang D, Chen J, Saraf A, Cassar S, Han P, Rogers JC, Brioni JD, Sullivan JP, Gopalakrishnan M: Association of Catsper1 or -2 with Cav3.3 leads to suppression of T-type calcium channel activity. *J Biol Chem* 2006;281:22332-22341.
- 32 Jagannathan S, Publicover SJ, Barratt CL: Voltage-operated calcium channels in male germ cells. *Reproduction* 2002;123:203-215.
- 33 Darszon A, Lopez-Martinez P, Acevedo JJ, Hernandez-Cruz A, Trevino CL: T-type Ca²⁺ channels in sperm function. *Cell Calcium* 2006;40:241-252.
- 34 Garcia MA, Meizel S: Progesterone-mediated calcium influx and acrosome reaction of human spermatozoa: pharmacological investigation of T-type calcium channels. *Biol Reprod* 1999;60:102-109.
- 35 Stamboulis S, Kim D, Shin HS, Ronjat M, De Waard M, Arnoult C: Biophysical and pharmacological characterization of spermatogenic T-type calcium current in mice lacking the Cav3.1 (alpha1G) calcium channel: Cav3.2 (alpha1H) is the main functional calcium channel in wild-type spermatogenic cells. *J Cell Physiol* 2004;200:116-124.