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Secretoneurin stimulates the production and release of luteinizing hormone in mouse L β T2 gonadotropin cells

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Abstract

Secretoneurin (SN) is a functional secretogranin II (SgII)-derived peptide that stimulates luteinizing hormone (LH) production and its release in the goldfish. However, the effects of SN on the pituitary of mammalian species and the underlying mechanisms remain poorly understood. To study SN in mammals, we adopted the mouse L β T2 gonadotropin cell line that has characteristics consistent with normal pituitary gonadotrophs. Using radioimmunoassay and real-time RT-PCR, we demonstrated that static treatment with SN induced a significant increment of LH release and production in L β T2 cells in vitro. We found that GnRH increased cellular SgII mRNA level and total SN-immunoreactive protein release into the culture medium. We also report that SN activated the extracellular signal-regulated kinases (ERK) in either 10-min acute stimulation or 3-h chronic treatment. The SN-induced ERK activation was significantly blocked by pharmacological inhibition of MAPK kinase (MEK) with PD-98059 and protein kinase C (PKC) with bisindolylmaleimide. SN also increased the total cyclic adenosine monophosphate (cAMP) levels similarly to GnRH. However, SN did not activate the GnRH receptor. These data indicate that SN activates the protein kinase A (PKA) and cAMP-induced ERK signaling pathways in the LH-secreting mouse L β T2 pituitary cell line.

Keywords: secretogranin II, gonadotropin-releasing hormone

AS ONE OF THE MAJOR GRANIN PROTEINS, secretogranin II (SgII) was initially characterized in bovine anterior pituitary (31). It is an ~600-amino acid, very acidic, tyrosine-sulfated protein located in secretory granules of vertebrate neuroendocrine cells (4, 8, 22, 26). Numerous small potentially bioactive peptides are derived from SgII precursor processing, but only the 33- to 34-amino acid segment, termed secretoneurin (SN), is conserved from fish to mammals (13, 48). In human pituitary, SgII immunoreactivity (IR) is localized to gonadotrophs, thyrotrophs, and corticotrophs (39). SgII IR was detected in the secretory granules and colocalized with LH in bovine gonadotroph, indicating the copackaging of granins and gonadotropins to form secretory granules (3). Two types of secretory granules were visualized in rat gonadotrophs: a large-sized moderately electron-dense granule and a small-sized electron-dense granule. The latter granule contained exclusively the immunoreactive signals of SgII and LH (41). In addition to the pituitaries of cow, rat and mouse, SgII was found in the LH-positive granules from female sheep gonadotrophs (6). The above immunocytochemical studies and other work on in vivo LH release (7) reveal an important association of SgII-related products and LH in

mammalian gonadotrophs.

Nicol et al. (28) utilized radioimmunoassay (RIA) to investigate protein release from the mouse L β T2 gonadotroph cell line. They observed that pulsatile GnRH treatments resulted in the marked increments of both LH and SgII release together at each time point, suggesting a close correlation between the secretions of these two proteins. Nicol et al. (29) also demonstrated that LH, but not follicle-stimulating hormone (FSH), is coreleased with SgII and chromogranin A (CgA) in response to GnRH in L β T2 cells, indicating that GnRH-stimulated LH secretion is via the regulated granin-associated pathway.

Our previous studies in the goldfish model revealed that SN not only stimulates the secretion and production of LH in vivo (4) and in vitro (45, 47, 50) but is also generated from proteolytic processing of SgII in the brain and pituitary (46, 47). Therefore, both neuroendocrine and paracrine sources of SN are involved in stimulation of gonadotroph function in the goldfish pituitary (50). We hypothesize that the well-conserved SN peptide also plays a role in regulating LH secretion in mammals. The mouse L β T2 gonadotroph cell line was chosen because it is a highly amenable model system that exhibits the major functional characteristics consistent with those of normal mammalian pituitary gonadotrophs (38) and produces SgII (28, 29), and intracellular signal transduction pathways are well described (14, 19, 20, 32, 40, 43). Our results show that SN stimulates intracellular signaling pathways similar to those regulated by GnRH. Given that mouse L β T2 cells also release SN-immunoreactive peptides, SN may be part of an paracrine and/or autocrine mechanism regulating LH release.

MATERIALS AND METHODS

Neuropeptides. The mammalian GnRH agonist [mGnRH-A (Des-Gly¹⁰, D-Leu⁶, Pro-NHET⁹)-LHRH] was purchased from Bachem Bioscience (King of Prussia, PA). The 33-amino acid mouse SN (mSN) peptide was synthesized on Fmoc-PAL-PEG polystyrene solid support by using an automated peptide synthesizer (Pioneer; PE-PerSeptive Biosystems, Framingham, MA) following HATU/DIEA-mediated Fmoc chemistry and purified by reversed-phase HPLC (2).

Culture and static incubation of L β T2 gonadotrophs. The L β T2 gonadotroph cell line was generously provided by Dr. P. Mellon (University of California, San Diego, CA). The culture method was described previously (38). Prior to each static incubation experiment, the cells were plated in 24-well culture plates at a density of $\sim 2.5 \times 10^5$ cells/well or six-well culture plates at a density of $\sim 2.0 \times 10^6$ cells/well and cultured in the 5% CO₂ air at 37°C for 48 h. At the beginning of experimentation, the medium was replaced with DMEM containing either 10 nM mGnRH-A or various doses of SN (1–100 nM). After the individual static treatments of 3, 6, and/or 12 h, depending on the experiment, media were collected and stored at –20°C for radioimmunoassay of LH. Moreover, cells were removed from the plate bottom and kept at –80°C for RNA or protein extraction. For the study of the signaling pathway, cells were starved in serum-free DMEM overnight in a wet 5% CO₂ atmosphere at 37°C at first before the static treatment of mGnRH-A (100 nM) and SN (10 and/or 100 nM) for 10 min or 3 h. For blocking the PKC-MAPK pathway, 20 μ M PD-98059 (a MEK inhibitor; Cell Signaling Technology, Danvers, MA) and 2 μ M bisindolylmaleimide I (BIM-1, a PKC inhibitor; Calbiochem, La Jolla, CA) were utilized to pretreat the L β T2 cells for 1 and 0.5 h, respectively, prior to the 10-min acute stimulation of mGnRH-A (100 nM) and SN (100 nM). PD-98059 and BIM-1 were chosen because they were shown to be effective in other experiments with L β T2 cells (19).

RIA. The LH levels in the culture medium were assayed by a mouse LH-specific RIA, as described previously, and validated using the L β T2 cell line (24, 28, 29). The intra- and interassay coefficients of variance were <10% for LH RIA. For the SN assay, 1 million L β T2 cells were treated with 10 nM mGnRH-A for 6 and 12 h, and the culture medium was lyophilized and resuspended in 150 μ l of RIA buffer. The SN RIA was performed as described (12). The RIA detects SN (SgII 154–186) and any larger protein derived proteolytically from SgII containing the SN sequence. The linear range of the RIA was 2–60 fmol. Expectedly, gel filtration analysis (16) of 60 fractions of L β T2 cell culture medium indicated that ~ 52 and 27% of the total SN IR was SN and the SgII precursor, respectively (data not shown).

RNA isolation, cDNA preparation, and real-time RT-PCR analysis. After a 6-h static incubation with mGnRH-A and SN, the L β T2 cells were carefully washed with 1 \times PBS. Total RNA was isolated using the RNeasy Micro Kit (Qiagen, Hilden, Germany), following the company's standard protocol. cDNA

synthesis and real time RT-PCR methods and validation steps were described previously by our laboratory (23). Briefly, after RNA levels were measured using the NanoDrop ND-100 Spectrophotometer (Thermo Scientific, Wilmington, DE), aliquots of 2 μ g of DNA-free RNA were used to synthesize cDNA with 200 ng of random primers (Invitrogen, Carlsbad, CA) and Superscript II RNase H⁻ reverse transcriptase (Invitrogen). The PCR primers were designed using Primer 3 (<http://frodo.wi.mit.edu>), verified by IDT OligoAnalyzer 3.1 (<http://www.idtdna.com/analyzer/Applications/OligoAnalyzer>), and synthesized by Invitrogen. Based on the published mouse sequences, the primer sets in the present investigation are LH β (NM_008497: forward 5'-CTGTCAACGCAACTCTGG-3', reverse 5'-AGGGCTACAGGAAAGGAG-3'), SgII (NM_009129: forward 5'-CTACCCTGGAGTCTGTGTTTC-3', reverse 5'-TTGCTGTCTCTCACCTCTTC-3'), CgA (NM_007693: forward 5'-AGCATCCAGTCCCCACTT-3', reverse 5'-AAGCCTCTGTCTTTCCATC-3'), and 18S ribosomal RNA (X00686: forward 5'-GATACCGTCGTAGTTCC-3', reverse 5'-ATCTGTCAATCCTGTCC-3') as a control for RNA loading. Depending on the sequence, amplification of cDNAs was performed with 150–300 nM of primers using the Mx3000 Multiplex Quantitative PCR System (Stratagene, La Jolla, CA).

Intracellular protein extraction and Western blot analysis. The L β T2 cells were removed from the six-well culture plates and collected by centrifuging at 2,000 rpm for 10 min. After these cells were sonicated in a homogenizing buffer, a protein extract was obtained from the supernatant and analyzed by Western blots, as described previously (47). Our anti-goldfish SN antiserum (dilution: 1:2,000–1:4,000) was generated against the 15-amino acid antigenic peptide YTPQKLATLQSVFEE, which is the most conserved central core of the SN sequence between various vertebrate species (46). The antibody specifically recognizes SN in fish and mammals, including human (46, 48). The anti-actin antibody (A2066, dilution: 1:500; Sigma, St. Louis, MO), phospho p44/42 MAP kinase (ERK1/2; Thr²⁰²/Tyr²⁰⁴) antibody (9101S, dilution: 1:600–1:1,000; Cell Signaling Technology), and p44/42 MAPK (ERK1/2) rabbit antibody (9102, dilution: 1:1,000; Cell Signaling Technology) were purchased from the indicated suppliers. Both MAPK antibodies were previously validated for use on L β T2 cells (20).

Measurement of total cAMP level. The L β T2 cells was preincubated with serum-free DMEM for 15 min and then changed to DMEM with 100 nM mGnRH-A and/or 100 nM SN and incubated for 0.5 h. The intracellular and extracellular cAMP level was determined as described previously (10, 17, 36). Total cAMP level is the sum of the intracellular and extracellular cAMP levels.

GnRH receptor activation assay. Human embryonic kidney (HEK)-293 cells transfected with a GnRH receptor (GnRHR) reporter construct were used to test the possibility that SN could activate the GnRHR. The experimental protocol was described in detail previously by Oh et al. (30). The cellular transfection was performed using the SuperFect transfection kit (Qiagen, Chatsworth, CA), 100 ng of rat GnRHR cDNA that was constructed at *EcoRI* and *XbaI* sites of pcDNA3 (Invitrogen, San Diego, CA), and 200 ng of SRE-luc (luciferase) vector [containing a single copy of the serum response element (SRE) CCATATTAGG followed by c-fos basic promoter] along with 200 ng of the internal control plasmid pCMV-Gal. The cells were treated with natural mGnRH and/or rat SN (Anygen, Gwangju, South Korea) for 6 h, and then the luciferase activity in the cell extract was determined.

Data analysis. Data were expressed as means \pm SE. The statistical analyses were carried out using Sigma Stat version 3.5. The effects of SN and mGnRH-A on LH release and mRNA levels of LH β , FSH β , SgII, and CgA were analyzed using one-way analysis of variance (ANOVA) followed by the Fisher least significant difference (LSD) test. When the values were nonparametric, data were naturally log-transformed before one-way ANOVA. When examining the effect of 10 nM mGnRH-A on SgII processing and SN IR protein release, we utilized either the *t*-test (for parametric data) or the Mann-Whitney rank sum test (for nonparametric data) to compare control and mGnRH-A-treated groups. The phospho-ERK1/2 and total ERK1/2 data were normalized by the average signal intensity for each membrane. The ratios between normalized phospho-ERK1/2 and total ERK1/2 levels were examined by one-way or two-way ANOVA followed by Fisher LSD test to determine specific differences among treatments. If the data were nonparametric, the Kruskal-Wallis one-way ANOVA on ranks was used. Means were considered significantly different if $P < 0.05$. Results are presented as means \pm SE. The fold change represents the ratio of the means of two groups of data (i.e., treatment over control/basal).

RESULTS

Dose response and time course studies of SN's effect on LH release. Various concentrations (1, 10, and 100 nM) of SN were chosen for the time course and dose response study of LH secretion from L β T2 cells. The levels of LH release to the culture medium increased from 3 to 12 h in the control group as well as in the mGnRH-A and SN treatments. We adopted 10 nM of mGnRH-A as the positive control in this experiment (28). After 3 h of treatment, mGnRH-A induced a 3.1-fold increment of LH levels ($P < 0.001$) compared with the control (Fig. 1). The LH levels after the exposure to 1 and 10 nM SN were 2.9- ($P < 0.001$) and 2.6-fold ($P < 0.01$) higher, respectively, than the time-matched controls. After 3 h, the LH level in the 100 nM SN group was increased 1.6-fold, but this did not reach statistical significance ($P > 0.05$). After a 6-h incubation, LH increased 3.4-fold ($P < 0.001$) in response to mGnRH-A; exposures to all SN doses (1–100 nM) enhanced ($P < 0.05$) LH levels in culture medium 2.9- to 2.7-fold (Fig. 1). In contrast, the effects of mGnRH-A and SN were no longer evident after 12 h (Fig. 1).

Expression of LH β , SgII, and CgA in L β T2 cells. Another experiment was carried out to explore the effects of SN on LH β -subunit mRNA levels. Cellular 18S ribosomal RNA levels were not significantly modified under any treatment (data not shown), and thus it was chosen as an internal standard to normalize the expression of LH β -subunit and other genes. In the dose response study (Fig. 1), there was no significant difference between 1- and 10-nM SN effects on stimulating LH release after either 3 or 6 h. Previous studies using goldfish pituitary cells in vitro (45, 49) indicated that 10 nM goldfish SN consistently enhanced LH production, so we examined a similar dose range (1, 10, 100 nM) of mouse SN to investigate SN-induced LH β gene expression in L β T2 cells. In the presence of 10 nM mGnRH-A, the LH β mRNA level was increased twofold ($P < 0.001$) compared with the control. This result was consistent with previous findings using L β T2 cells (38). Treatments of 1–100 nM SN induced 1.8- to 1.6-fold ($P < 0.05$) increments in LH β gene expression (Fig. 2A). Furthermore, SN-induced LH β mRNA levels exhibited a pattern similar to that of LH secretion after 6 h, which is shown in Fig. 1. Mammalian GnRH-A-induced LH β mRNA in cells and LH levels in the culture medium were similar to the effect of SN. Treatment with mGnRH-A also upregulated SgII mRNA levels 2.4-fold ($P < 0.001$) compared with the control. In contrast, SgII mRNA was not affected by any dose of SN during the 6-h static incubation of L β T2 cells (Fig. 2B). Compared with SgII expression, CgA mRNA levels were not affected by mGnRH-A and were somewhat reduced by 1 (57%, $P < 0.001$) and 10 nM (71%, $P < 0.05$) SN in the 6-h-treated cells (Fig. 2C).

Evaluation of anti-goldfish SN antiserum for the detection of mSN. Our anti-goldfish SN antiserum was generated against the 15-amino acid antigenic peptide YTPQKLATLQSVFEE, which is the most conserved part of the SN sequence between various vertebrate species (46). The antigenic peptide shows 80% identity to the middle portion of mSN (48), indicating the possibility of using the anti-goldfish antibody to examine the SN IR in mice. In addition, our anti-goldfish SN antibody was also used to detect the SN-IR signals within the pituitary cells in human, rat, and trout (48, 49). To evaluate the specific binding ability of anti-goldfish SN antibody to the protein extracted from mouse L β T2 pituitary cells, we carried out Western blot analysis using anti-goldfish SN antiserum preabsorbed with its antigenic peptide goldfish SN (Fig. 3). We detected several SN-immunoreactive protein bands when using the anti-goldfish SN antiserum (Fig. 3A, lane I), whereas no signal was observed in the membrane that was incubated in the SN antibody preabsorbed with SN (Fig. 3A, lane II). These results confirmed that the SN antiserum recognized SN-associated proteins derived from mouse pituitary cells.

Under a longer enhanced chemiluminescence exposure time than that of Fig. 3A, two strong high-molecular-weight (MW) bands and two weak low-MW signals were observed in different samples (Fig. 3B). A standard formula ($y = 116.65x^{-0.7378}$, $r^2 = 0.999$) was established for describing the relationship between apparent MW (y) and gel running distance (x) based on protein standards. The estimated MWs of the two strong and two weak bands were ~ 71 , ~ 46 , ~ 42 , and ~ 32 kDa (Fig. 3B). The ~ 71 -kDa band is corresponding to the size of the unprocessed mouse SgII precursor. The mouse SgII sequence is 617 amino acids with a calculated MW of 70.6 kDa. Expectedly, the other SN-immunoreactive signals were noticeably smaller than the calculated MW of the SgII precursor, indicating that they are processing products derived from this granin.

Effects of 6- and 12-h mGnRH-A treatments on SgII-immunoreactive products in mouse L β T2 pituitary cells. We focused on GnRH regulation of SgII products. After a 6-h static incubation, 10 nM mGnRH-A

significantly decreased the cellular levels of SgII, ~46-kDa, and ~32-kDa SN-immunoreactive polypeptides by 49 ($P = 0.05$), 28 ($P < 0.001$), and 37% ($P = 0.05$), respectively, and also induced a 36% but statistically nonsignificant decrease ($P = 0.505$) in the level of ~42-kDa, SN-immunoreactive, SgII-derived peptide (Fig. 4, A and B). Twelve-hour treatment of mGnRH-A (10 nM) also modulated the levels of all SN-immunoreactive peptides. The mGnRH-A treatment decreased the ~71-, ~46-, ~42-, and ~32-kDa SN-containing proteins by 63 ($P < 0.01$), 31 ($P < 0.001$), 41 ($P < 0.05$), and 49% ($P = 0.05$), respectively (Fig. 4, C and D). Note that β -actin served as the protein-loading control and did not change with treatments (Fig. 4, B and D). Furthermore, 10 nM mGnRH-A increased radioimmunoassayable SN IR in the culture medium level 3.0- ($P < 0.05$) and 2.3-fold ($P < 0.001$) after 6 and 12 h, respectively (Fig. 5, A and B).

SN activates the PKC-MAPK cascade in L β T2 cells. ERK is a classic MAP kinase involved in GnRH-stimulated LH production and release in L β T2 cells (32). In this experiment, Western blot analysis was used to detect the changes of active phospho-ERK1/2 and total inactive ERK1/2 levels under various treatments. At first, we examined the acute effect of SN on the activation of ERK1/2. Serum-starved L β T2 cells were treated with 100 nM mGnRH-A 10 and 100 nM SN for 10 min. As a positive control group, the treatment of 100 nM mGnRH-A increased activated ERK1/2 3.0-fold ($P < 0.001$; Fig. 6A), which is consistent with the data described previously by others (19). Similar to the action of mGnRH-A, 10 and 100 nM SN were capable of inducing 2.2- ($P < 0.01$) and 4.6-fold ($P < 0.001$) rapid increments, respectively, of phospho-ERK1/2 levels (Fig. 6A). The 100-nM SN-stimulated phospho-ERK1/2 increment was significantly higher than that of 100 nM mGnRH-A by 1.6-fold ($P = 0.001$; Fig. 6A). When L β T2 cells were exposed to the low-dose treatment for a longer time (3 h), 10 nM SN also significantly increased the phospho-ERK1/2 levels 1.4-fold ($P < 0.05$; Fig. 6B). To further define the signaling pathways involved in activation of ERK by SN, the cells were pretreated with PD-98059 and BIM-1 to inhibit MEK and PKC, respectively, two major kinases upstream of ERK. We compared the relative change of activated ERK1/2 after the 10-min stimulation with 100 nM SN and 100 nM mGnRH-A. PD-98059 significantly reduced both the mGnRH-A- and SN-induced activation of ERK1/2 by 48 ($P < 0.05$) and 40% ($P < 0.01$), respectively (Fig. 6C). BIM-1 also significantly ($P < 0.001$) reduced both the mGnRH-A- and SN-induced activation of ERK1/2 by 96 and 76%, respectively (Fig. 7A).

SN regulates the cAMP levels of L β T2 cells. After 0.5-h static treatments of mGnRH-A (100 nM) and SN (100 nM), the total cAMP levels were measured for L β T2 cells. As depicted in Fig. 7B, both mGnRH-A and SN significantly increased the total cAMP levels 1.4- ($P < 0.05$) and 1.3-fold ($P < 0.05$).

SN is not able to activate the GnRHR. In HEK-293 cells transfected with the rat GnRHR, natural mGnRH significantly increased SRE-luc activity over the range of 1–1,000 nM (Fig. 8), confirming previous data using the same reporter system (30). However, rat SN (1–1,000 nM) did not affect SRE-luc activity (Fig. 8A). Moreover, there were no interactions between mGnRH and SN; treatment with 10 μ M SN did not alter ($P > 0.05$) the SRE-luc response to 100 nM mGnRH (Fig. 8B).

DISCUSSION

Our previous studies in the goldfish model demonstrated that SN has neuroendocrine and paracrine effects to stimulate LH secretion and production in dispersed pituitary cells (47, 50). We have suggested that the elevation of intracellular Ca^{2+} level is a potential transduction signal mediating SN action on goldfish LH cells. This SN-induced Ca^{2+} signaling mechanism was also demonstrated to be partly independent of GnRH-stimulated Ca^{2+} entry (47). However, the SN signaling pathway in gonadotrophs is still unclear. Therefore, we chose to study SN action on the L β T2 cell. They have many of the common characteristics of normal mouse pituitary cells, and the signaling pathways to mediate GnRH-stimulated LH release are well studied (32). In agreement with our previous research in goldfish (47), the present study revealed that SN increased not only LH secretion but also the mRNA levels of the LH β -subunit in mouse L β T2 cells. Therefore, the effect of SN on LH appears to be evolutionarily conserved and indicates for the first time a potential regulatory role for SN in mammalian reproduction.

Various studies in the goldfish have shown that SN induces LH release and LH β -subunit gene expression in pituitary cells (4, 45, 47). However, studies concerning the regulatory effects of SN in mammalian neuroendocrine and central nervous systems are quite limited. Secretoneurin was demonstrated to dose-dependently increase dopamine outflow from superfused rat striatal slices (34). An in vivo experiment

showed that local infusion of SN into the substantia nigra and neostriatum of halothane-anesthetized rats elevated the extracellular concentrations of dopamine, γ -aminobutyric acid, and glutamate (44). Here, we adopted mouse L β T2 gonadotrophs to study the effects of SN on LH release. In this cell line, there is a close correlation between GnRH-induced LH release and secreted SN-immunoreactive SgII-like proteins. A possible regulatory effect of SgII (or derived peptides) on LH secretion from L β T2 gonadotrophs had been proposed previously (28). We found that the effective concentrations of SN to stimulate LH release in vitro from both L β T2 gonadotrophs and goldfish pituitary fragments are similar (45). A similar dose range and effect of SN were also observed in primary cultures of dispersed goldfish pituitary cells. Six-hour static treatment of 10 nM SN significantly increased LH secretion from gonadotrophs (47). We conclude that low physiologically relevant nM doses of SN are able to stimulate LH secretion from pituitary cells.

During the 3- and 6-h static cultures, the L β T2 cells were very sensitive to even the lowest dose of SN (1 nM) tested. Similar to the effect of SN in L β T2 cells, a low-dose pulse of 10 nM GnRH could induce more LH release than 50 and 200 nM GnRH in the same cell line (28). Unlike L β T2 gonadotrophs, which are tumor derived, dispersed normal mouse pituitary cells show a classical LH response to increasing doses of GnRH after 4-h static treatments over a wide range of GnRH concentrations (10–1,000 nM) (1). This difference between L β T2 gonadotrophs and normal pituitary cells is likely due to alteration of secretory pathways, a well-known characteristic of the highly selected L β T2 cell line. A previous investigation revealed that 10 and 100 nM GnRH evoked a similar increment in exocytosis as well as intracellular Ca²⁺ level within a single L β T2 cell (37). These authors' interpretation of the result was that only ~400 vesicles were found to be involved in fusion with the plasma membrane for further secretion. Thus, secretion under the long-term stimulation of the L β T2 cell might be highly limited by the availability of secretory products (37). The secretory limitation was also observed in long-term GnRH-evoked LH release; the LH secretory response declined dramatically under the repeated stimulation of GnRH for several hours in L β T2 cells (38). In contrast, multiple administrations of GnRH to perfused normal rat pituitary cells always induced dose-dependent LH release (21). It is likely that the absence of a dose response to SN-induced LH release over the dose range tested results from this limitation of secretory vesicles in the mouse L β T2 cell line. Nevertheless, we show for the first time that SN stimulates LH release from mouse pituitary cells in a manner similar to that which we have documented for normal goldfish pituitary cells in vitro.

To further examine the effects of SN on LH generation, we demonstrated that the increasing doses of SN induced similar increment in LH β gene expression within L β T2 gonadotrophs, consistent with the pattern of SN-induced LH release. An unvaried LH synthesis under increasing doses of SN treatments might be an alternative explanation for the absence of a dose-dependent effect of SN on LH secretion in L β T2 cells. Interestingly, the levels of SN-induced LH release (2.6- to 2.8-fold increase) were higher than those of LH β -subunit mRNA (1.6- to 1.8-fold increment). SN might affect not only LH β mRNA synthesis but also other pathways associated with LH protein generation and secretion. This possibility has been suggested but never tested in a previous study using this cell line (28). The acute GnRH-induced LH protein synthesis and secretion were dependent mainly upon the activation of translation initiation proteins 4E-binding protein 1 and eukaryotic initiation factors 4E/4G but not LH β transcription. The stimulation of these proteins by GnRH was mediated by the MAPK/ERK pathways (27). The stimulatory effects of SN on the MAPK cascade in the L β T2 cell line (this study) indicate the possibility of multiple pathways mediating SN-induced LH release.

In comparison with pulsatile GnRH stimulation that increased the secretion of both SgII and CgA from perfused L β T2 cells (28), we determined that static exposure to mGnRH-A also increased the cellular level of SgII mRNA but had no remarkable effect on CgA gene expression. However, Nicol et al. (28) reported that the pulses of GnRH did not alter SgII mRNA level in estradiol- and dexamethasone-treated L β T2 cells. The variation between our data and early observations might be attributed to differences in GnRH stimulation strategies and the different in vitro models adopted. Under the same conditions as the GnRH treatment, SN was unable to elicit any changes in SgII mRNA levels in our experiment with L β T2 cells. In contrast, SN significantly decreased CgA mRNA levels at the doses of 1 and 10 nM. These data indicate that, under the in vitro conditions described, mGnRH-A and SN are acting differentially on expression of SgII and CgA. They are both granins but from distinctly different molecular families (48).

Our previous studies of SN generation in goldfish revealed the formation of several SgII-derived proteins and polypeptides. In the pituitary, we detected proteins of molecular masses of \sim 57, \sim 30, \sim 19.6, \sim 15, and \sim 12 kDa in addition to the free SN peptide (molecular mass: 3,655 Da) (46, 47). Using a similar strategy, we discovered four SN-immunoreactive signals with distinct molecular masses predicted as \sim 71, \sim 46, \sim 42, and \sim 32 kDa in mouse L β T2 gonadotrophs. Moreover, the \sim 71- and \sim 46-kDa proteins showed significantly higher levels than the \sim 42- and \sim 32-kDa polypeptides. Some of the resultant processing products have sizes similar to those reported for other mammalian SgIIs (9, 11, 39). Almost all of the SgII precursor protein is processed to generate free SN in normal rat pituitary (18), and gonadotrophs secrete SN-immunoreactive products (Ref. 28 and this study).

It was demonstrated previously using RIA that GnRH decreased the cell content and increased the secretion of total SgII-related proteins from L β T2 cells (28). Under the conditions of in vitro culture, the speed of GnRH-induced SgII processing appears to be faster than SgII generation at some time points, which explains the observed decrease in cellular SgII-related proteins (28). Consistent with this, our Western blot analysis showed that GnRH was able to decrease cellular levels of SgII and its derived proteins. Moreover, GnRH also increased SN-immunoreactive protein release from mouse L β T2 pituitary cells and GnRH-increased SgII mRNA levels, indicating the potential for increased SgII precursor protein production. This result is similar to what we found in goldfish in vivo (33) and in vitro (50). These data indicate that GnRH enhances SgII synthesis and processing.

The MAPK cascade is one of the essential GnRH-activated G protein-coupled signaling pathways to induce early genes for gonadotropin production and release. The MAPK pathway includes mainly ERK, p38 MAPK, and c-Jun NH₂-terminal kinase (JNK) (32). The activated form of ERK is phosphorylated on threonine and tyrosine residues within the phosphorylation lip (5). In the L β T2 cell line, GnRH was demonstrated to rapidly activate ERK and p38 MAPK, but activation of JNK was weaker and slower (19). In agreement with this previous report, our observations confirmed that the 10-min acute mGnRH-A treatment caused the activation of ERK in L β T2 cells. Similarly to the stimulatory action of mGnRH-A, 10 and 100 nM SN also increase the ERK phosphorylation 2.2- and 4.6-fold, respectively. However, chronic GnRH exposure can suppress PKC- and cAMP-induced ERK activation in L β T2 cells (20). In another experiment, we found that long (3 h) exposure to a low dose (10 nM) of SN treatment still stimulated ERK phosphorylation, but this was weaker than that observed at 10 min. MEK, in association with GnRH-stimulated PKC and Raf, has been reported to stimulate activation of downstream ERK in L β T2 cells (19). We also found that the inhibition of MEK with PD-98059 reduced both GnRH-A and SN-induced ERK phosphorylation. This result indicates that SN signals through the ERK-dependent MAPK pathway.

PKC is the most important signaling molecule to connect the G protein-coupled signal transduction and the MAPK cascade for mediating GnRH-induced LH subunit gene transcription (25). Activation of ERK caused by GnRH-induced PKC was reported for rat pituitary cells (42) and the mouse α T3-1 gonadotropin cell line (35). Liu et al. (19) demonstrated that GnRH activated ERK and two other MAPK family members, JNK and p38 MAPK, via a PKC-dependent signaling pathway in L β T2 cells. We observed that SN-induced ERK activation was significantly blocked by pharmacological inhibitors of PKC and MEK. SN-regulated migration and number of mouse endothelial progenitor cells in the inflammatory system are also via ERK activation (15). A previous study also showed that cAMP is involved in activating ERK to mediate GnRH-induced LH generation in L β T2 cells (20). Moreover, cAMP is important for GnRH-regulated G protein-coupled PKA signaling transduction in L β T2 cells (32). Secretoneurin exposure elevated total cAMP production in L β T2 cells, providing additional evidence for a G protein-coupled signaling pathway. To exclude the possibility that SN binds to the GnRHR to activate these transduction pathways, we examined GnRHR-induced SRE-luc activities following SN treatments of transfected HEK-293 cells. In this sensitive system, SN did not activate the rat GnRHR, and SN did not interfere with GnRH action. These data support the proposal that the putative receptor-linking SN action to cAMP-dependent and PKC-MAPK signaling pathways and LH synthesis is distinct from the GnRHR.

In conclusion, results from the present study provide the first evidence for a direct stimulatory action of SN on LH release and LH β -subunit mRNA levels in mammalian pituitary cells. Furthermore, we have shown that SN activates ERK, which is likely involved in the stimulation of LH β -subunit transcription via multiple G protein-coupled signaling pathways, including the PKC-MAPK and cAMP-dependent

mechanisms, in a similar manner as that observed for GnRH actions on L β T2 cells. GnRH regulates SN-immunoreactive peptides derived from SgII in the gonadotroph, suggesting that endogenous SN is a participant in an autocrine mechanism under the control of hypothalamic hormones. The studies of SN production, biological activities, and functional mechanisms in a mouse gonadotroph cell line support our hypothesis that SN is an evolutionarily conserved hormonal peptide involved in the regulation of vertebrate reproduction (45, 47, 50). To our knowledge, the clearest example of an endocrine role for SN is the stimulatory effects on LH release that we report in both fish and mammalian model systems. We suggest that SN activates a G protein-coupled receptor in gonadotrophs. Future investigations must focus on isolating the SN receptor, which has yet to be identified in any species.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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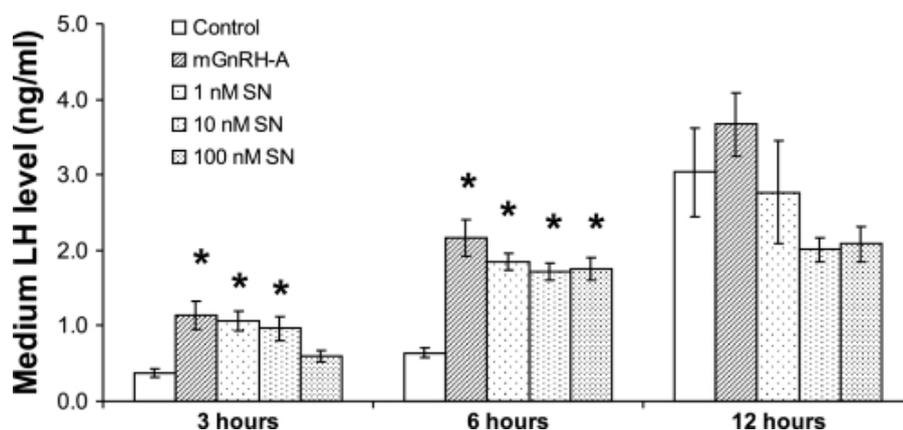
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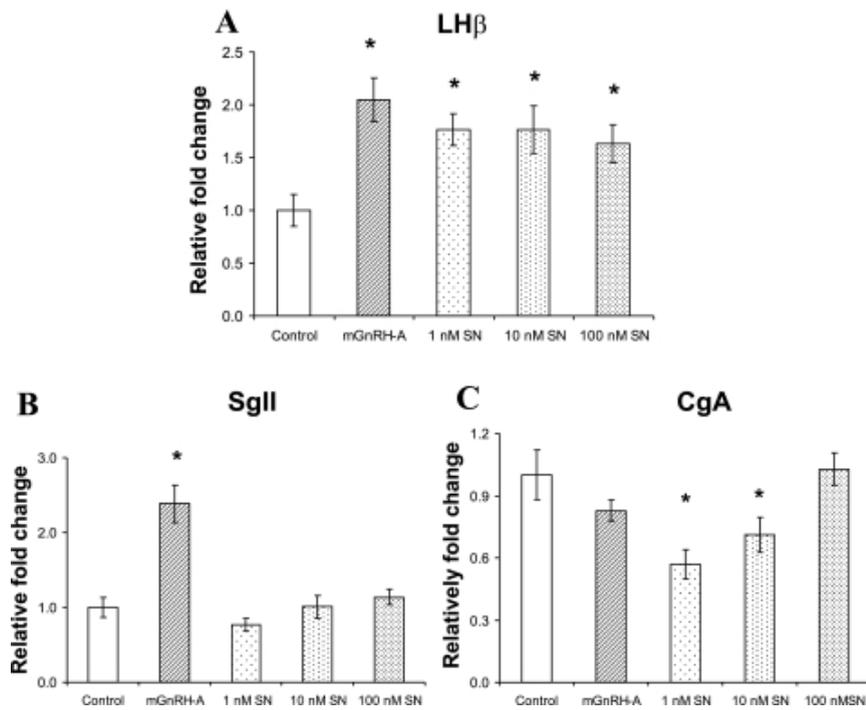
Figures and Tables

Fig. 1.



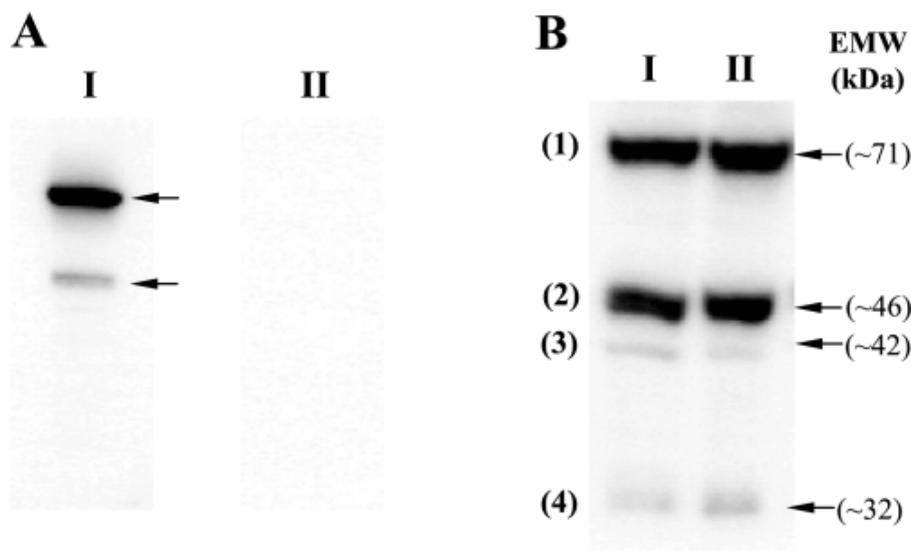
Time- and dose-dependent effects of secretoneurin (SN; 1, 10, and 100 nM) and mammalian GnRH agonist (mGnRH-A; 10 nM) after 3-, 6-, and 12-h static incubation on LH secretion from the mouse L β T2 pituitary cells. Results are presented as means \pm SE ($n = 9-10$). One-way ANOVA was chosen to assess the difference between control and treatment at a given time point, followed by Fisher least significant difference (LSD) test. Natural logarithms were adopted if data were not normally distributed in statistics. *Significant difference from control at a given time point ($P < 0.05$).

Fig. 2.



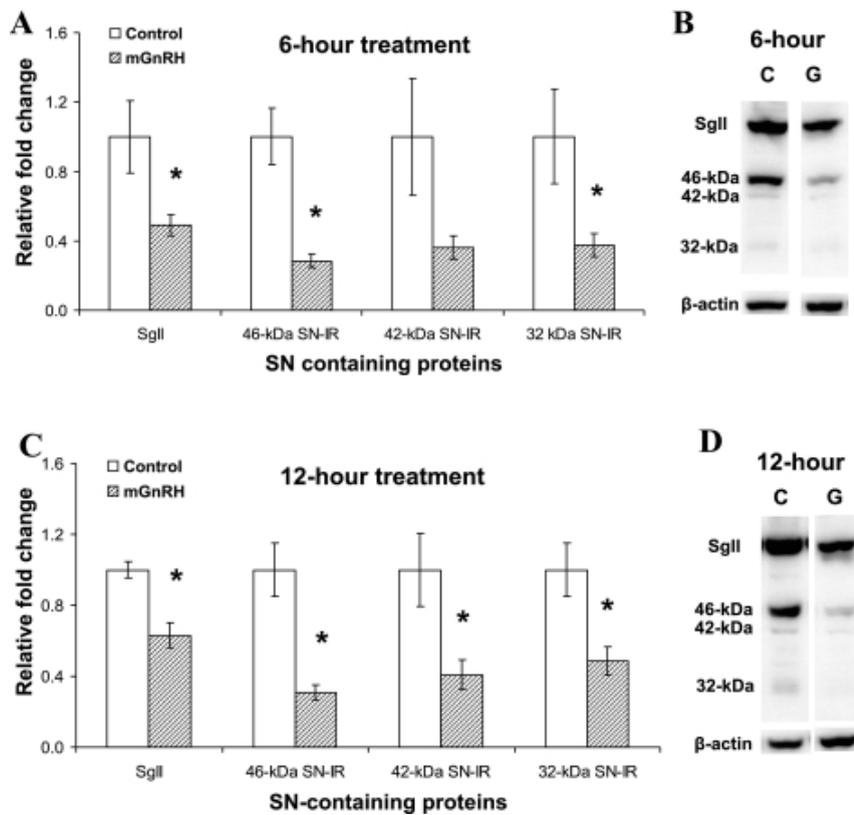
Assessment of the gene expression changes of LH β (A), secretogranin II (SgII; B) and chromogranin A (CgA; C) in L β T2 cells after 6-h static incubation with treatments of 10 nM mGnRH-A and 10 nM SN. The mRNA level was measured by real-time RT-PCR; 18S ribosomal RNA was used as an internal standard for sample loading control. One-way ANOVA was chosen to assess the difference between control and treatment, followed by Fisher LSD test. Natural logarithms were adopted if data were not normally distributed in statistics. Values are expressed as mean folds of control \pm SE. *Significant difference relative to control ($P < 0.05$).

Fig. 3.



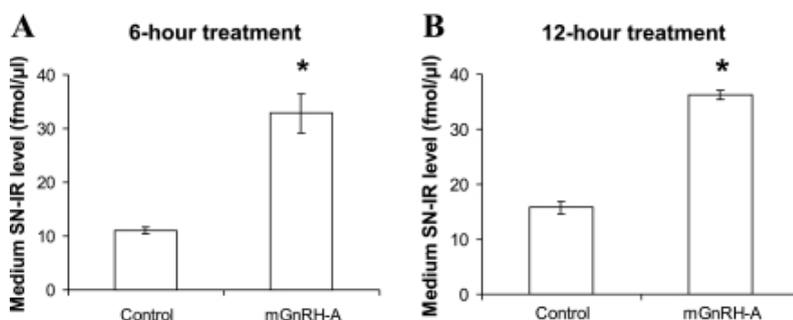
Western blotting analysis of the total proteins extracted from mouse L β T2 cells using anti-SN antibody (1:2,000–1:4,000). A: 30 μ g of protein extracts was separated by 10% SDS-PAGE gel and detected by anti-goldfish SN antiserum (46). Lane I: detection using anti-SN antibody (1:4,000); lane II: detection using anti-SN antibody (1:4,000) preabsorbed by 1 μ M goldfish SN. B: the loading samples of lanes I and II are taken from different culture wells. Arrows indicate 4 SN-immunoreactive signals that are ~71, ~46, ~42, and ~32 kDa. Their estimated molecular weights (EMW) are calculated from a standard curve and a formula depicting the relation between molecular weight of biotinylated protein standards and gel running distance.

Fig. 4.



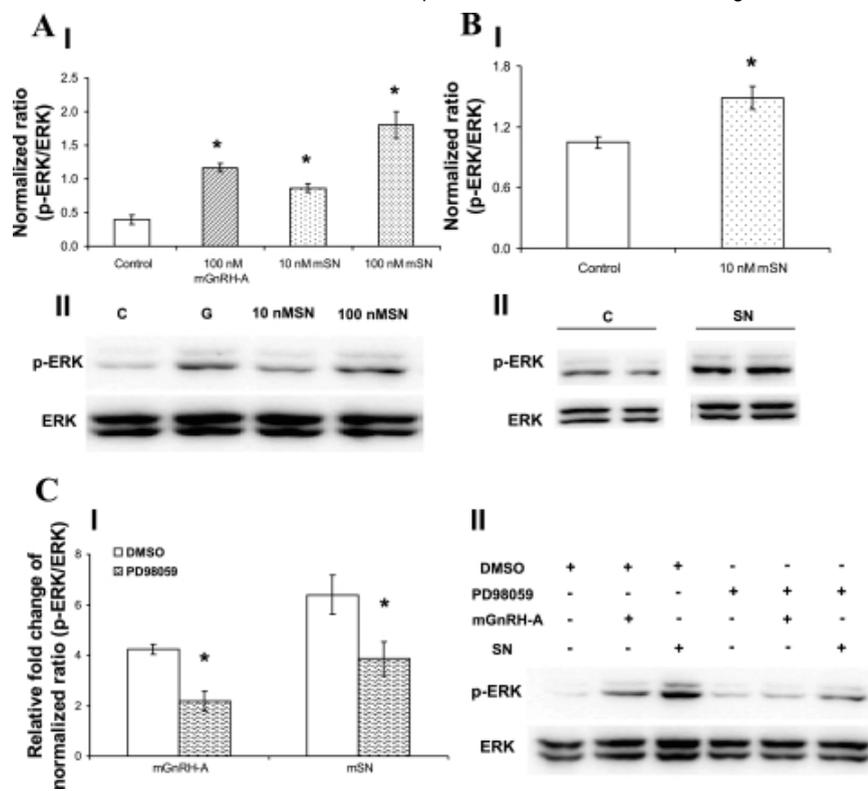
Effects of mGnRH-A (G) on intracellular content of SgII and derived peptides containing the SN sequence in L β T2 cells. *A* and *B*: levels of SN-immunoreactive (IR) proteins (~71, ~46, ~42, and ~32 kDa) were analyzed by Western blots using anti-SN antibody (dilution 1:2,000) after 6-h treatment of 10 nM mGnRH-A. *C* and *D*: the expression changes of SN-IR proteins under 12-h static incubation of mGnRH-A (10 nM). β -Actin was measured as an internal standard for loading control (C). Note that no signal was observed on membranes that were incubated in the SN antibody preabsorbed (4°C for 18 h) with 1 μ M SN (not shown), confirming previously reported specificity (46, 48). For *A* and *C*, results were presented as means \pm SE of the relative expression of SN-IR; $n = 8$ for both experiments. Western blots of SN and β -actin were preformed on the same membrane. The results of ~42- and ~46-kDa protein intensities and SN-IR protein release after 12-h treatment were parametric, and the rest of the data were nonparametric. When the data were nonparametric, Mann-Whitney rank sum test was used instead. *Significant difference between treatment and control ($P < 0.05$).

Fig. 5.



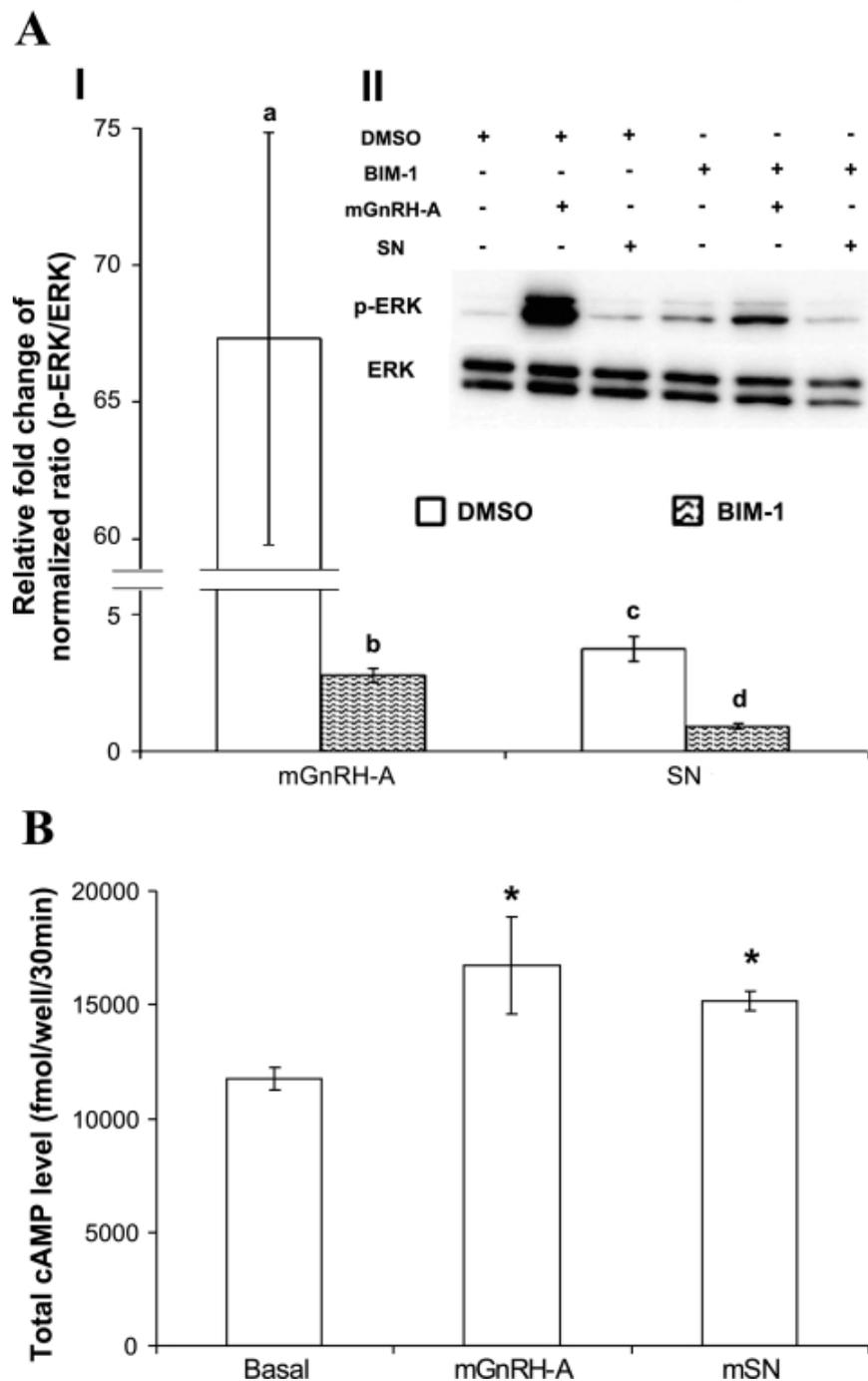
Ten nanomolars of mGnRH-A stimulates the release modification of SN-IR protein release into culture medium after 6 (*A*) and 12 h (*B*). Note that Western blots of SN and β -actin were preformed on the same membrane. A *t*-test was used to compare control and mGnRH-A groups. *Significant difference between treatment and control ($P < 0.05$).

Fig. 6.



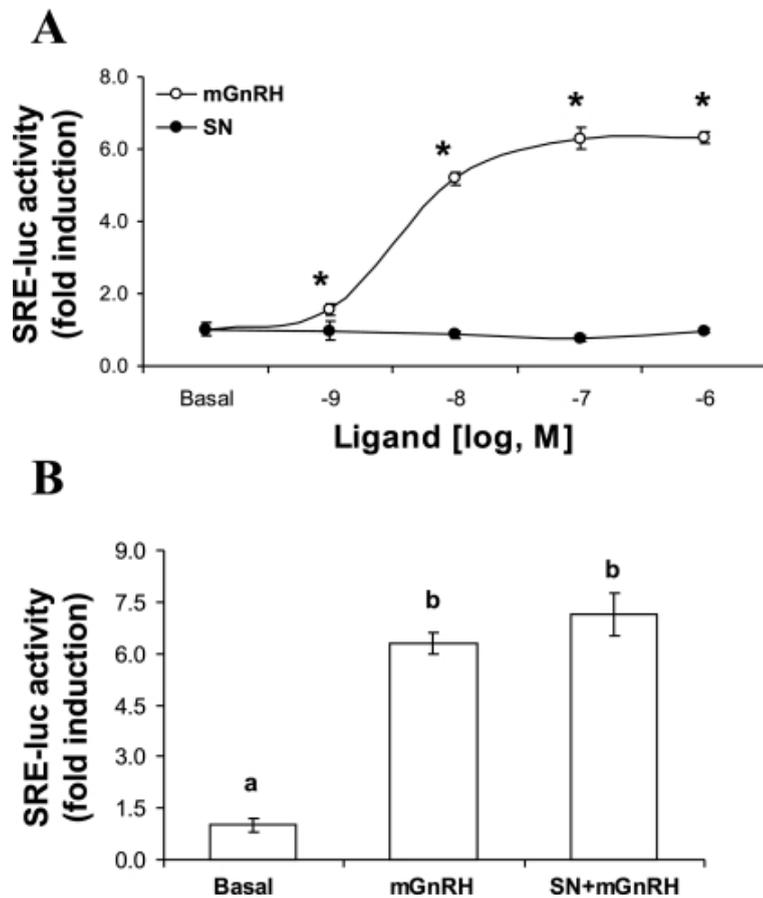
Action of SN to stimulate ERK1/2 in L β T2 cells. **A**: both mGnRH-A (100 nM) and SN (10 and 100 nM) stimulated ERK phosphorylation. These cells were cultured in serum-free DMEM overnight and stimulated by mGnRH-A and SN for 10 min. Protein extracts were first-analyzed Western blots with the antibody for phosphorylated ERK1/2 (p-ERK; *II, top*). After p-ERK1/2 blots were stripped, the membranes were immunoblotted again with the total ERK1/2 antibody (*II, bottom*). In *I*, data were presented as means \pm SE of the normalized ratio between p-ERK1/2 and total ERK1/2; $n = 5$. One-way ANOVA was chosen to assess the difference from control, followed by Fisher LSD test. *Significant difference between treatment and control ($P < 0.01$). **B**: SN (10 nM) activated ERK after 3 h. Cells were stimulated with a low dose of SN for 3 h in the static incubation. Western blots were used to assess p-ERK1/2 (*II, top*) and total ERK (*II, bottom*). The normalized ratio of 3-h-induced p-ERK/total ERK is shown as means \pm SE in *I*. *Statistical difference ($P < 0.01$, t -test; $n = 5$). **C**: PD-98059 (20 μ M, a MEK inhibitor) attenuated ERK1/2 phosphorylation. L β T2 cells were cultured in serum-free medium overnight and pretreated with 0.1% DMSO (the PD-98059 vehicle) and 20 μ M PD-98059 for 1 h and then stimulated by mGnRH-A (100 nM) and mouse SN (mSN; 100 nM), respectively, for 10 min. Cellular extracts were separated by 10% SDS-PAGE gel and detected by p-ERK and ERK1/2 antibodies. In *I*, relative change of normalized ratio (p-ERK/ERK) with respect to the group-matched control value was shown as means \pm SE ($n = 6$). * $P < 0.05$ vs. treatment without the MEK inhibitor PD-98059. The statistical difference was analyzed by 2-way ANOVA followed by Fisher LSD method. In *II*, the changes of signal intensities for p-ERK1/2 (*top*) and total ERK1/2 (*bottom*) were exhibited. Note that Western blots of ERK and p-ERK were performed on the same membrane.

Fig. 7.



A: bisindolylmaleimide (BIM-1; a PKC inhibitor) reduced ERK1/2 phosphorylation. L β T2 cells were cultured in serum-free DMEM overnight and pretreated with 0.1% DMSO (BIM-1 vehicle) and 2 μ M BIM-1 for 0.5 h and then stimulated by mGnRH-A (100 nM) and SN (100 nM), respectively, for 10 min. Cellular extracts were separated by 10% SDS-PAGE gel and detected by p-ERK1/2 and ERK1/2 antibodies. In *I*, relative change of normalized ratio (p-ERK/ERK) with respect to the group-matched control value was shown as means \pm SE ($n = 4$). ^{a-d}Different letters represent statistical differences ($P < 0.001$ vs. treatment without BIM-1). The statistical difference was analyzed by 2-way ANOVA, followed by Fisher LSD method. In *II*, the changes of signal intensities for phospho-ERK1/2 (*top*) and total ERK1/2 (*bottom*) were exhibited. **B:** the effects of SN (100 nM) and mGnRH-A (100 nM) on the total cAMP levels of L β T2 cells after 0.5 h. Results presented means \pm SE; $n = 6$. One-way ANOVA was chosen to assess the difference between basal and treatments. The data were nonparametric and the Student-Newman-Keuls method was used for analysis. *Significant difference relative to control ($P < 0.05$). Note that Western blots of ERK and p-ERK were performed on the same membrane.

Fig. 8.



Lack of SN activity on rat gonadotropin-releasing hormone receptor (GnRHR). human embryonic kidney-293 cells were transiently transfected with 100 ng of rat GnRHR and 200 ng serum response element (SRE)-luc reporter construct. *A*: the SRE-driven transcriptional activity in response to various concentrations of 1–1,000 nM (10^{-6} – 10^{-9} mol/l) for natural mGnRH and rat SN. * $P < 0.001$ vs. the basal SRE-luc activity ($n = 3$). Data were analyzed by 2-way ANOVA followed by Fisher LSD method. *B*: mGnRH (100 nM)-induced GnRHR activity in the presence or absence of rat SN (10 μ M). Data are shown as means \pm SE ($n = 3$). One-way ANOVA followed by Fisher LSD method was used to assess the difference between basal and treatments. ^{a,b}Different letters indicate that means are significantly different from each other ($P < 0.001$).

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