Induced Synaptotagmin (SYT) Protein Family Binds to Membrane SNAP25 to Facilitate Vesicle Secretion in Murine Granulosa/Cumulus Cells during Ovulation

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Abstract: *During ovulation, granulosa cells and cumulus cells synthesize and secrete a wide variety of factors via the exocytosis system. Exocytosis is controlled by the SNARE (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor) complex consisting of proteins residing in the vesicle membrane and the plasma membrane. SNAP25 localizes on the plasma membrane whereas synaptotagmins (SYT) reside in secretion vesicle membranes. After Ca^{2+}-induced binding of SNAP25 and SYT, the release of vesicle-contained factors is triggered. Therefore, we sought to determine if induction of Syt mRNA and binding of SYT to SNAP25 in granulosa and cumulus cells were involved in exocytosis during ovulation. After hCG stimulation, the expression levels of Syt1, Syt2, Syt4 and Syt6 were induced markedly in granulosa cells, whereas the levels of Syt3 and Syt7 mRNAs did not change dramatically. The levels of SYT1 protein were also increased 4 hr after hCG injection and remained increased until 16 hr post-hCG. Immunofluorescence using an SYT1 specific antibody showed increased staining for SYT1 in the granulosa cells and in the cumulus cells of hCG-primed mice. Additionally, the SYT1 antibody coimmunoprecipitated SNAP25 in granulosa cell lysates collected at 8-16 hr post-hCG. From these results, we conclude that the interactions of SYT1 and SNAP25 facilitate the exocytosis system in granulosa cells and cumulus cells during ovulation.*

Key words: *Exocytosis, Ovulation, Granulosa cells, SNARE, Synaptotagmin*

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Introduction

The pituitary surge of luteininzing hormone (LH) acts on the granulosa cells of preovulatory follicles to terminate the follicular program while at the same time stimulating the expression of genes required for ovulation and luteinization [1, 2]. DNA microarray and protein array systems have identified the secreted factors that are expressed in granulosa cells and in cumulus cells following LH/hCG stimulation. One group of secreted factors in the Interleukin (IL) family, including IL-1β, IL-6, IL-12, IL-17 and IL-18, which are expressed in granulosa cells and in cumulus cells during ovulation [3–5]. Addition of IL-1β to LH-containing medium significantly increases the number of oocytes ovulated from *in vitro*-perfused ovaries, whereas this is completely suppressed by an IL-1 receptor antagonist [6]. It has been demonstrated that IL-6 regulates hyaluronan synthesis and accumulation within the cumulus cell matrix [7]. The receptor GP130 is also expressed in cumulus cells and in granulosa cells [8]. Thus, secretion of cytokines from cumulus cells and granulosa cells, especially IL-1β or IL-6, is required for oocyte maturation, ovulation and fertilization.

The process of secretion of cytokines is complex but

is known to be controlled by SNARE (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor), which is composed of SNARE proteins residing in the vesicle membrane (synaptobrevin) and plasma membrane (syntaxin and SNAP25) [9, 10]. SNAP25 is anchored in the plasma membrane by palmitoylation of four cysteines residing in the linker region between the two SNARE motifs. In our previous study [5], we showed that SNAP25 is expressed in granulosa cells and in cumulus cells during ovulation. Knockdown of *Snap25* gene expression by the siRNA technique significantly suppresses secretion of cytokines from granulosa cells, whereas the transfection does not affect their mRNA levels [5], suggesting that SNAP25 is involved in the exocytosis system in granulosa cells and in cumulus cells during ovulation.

After increase of the Ca^{2+} level in the cells, the secreted vesicle binds to SNAP25 to trigger the release of vesicle-contained factors [11, 12]. The synaptotagmin (SYT) family is known as a Ca^{2+} censor and localizes on secreted vesicles [13, 14]. Most SYT family members have a Ca^{2+} binding domain and bind to SNAP25 in a Ca^{2+}-dependent manner [12]. Other members without a Ca^{2+} binding domain that bind to SNAP23 are involved in Ca^{2+}-independent exocytosis (non-regulated exocytosis) [15]. Thus, SYT family members plays an important role in Ca^{2+}-induced SNAP25 dependent exocytosis however, there is little information available concerning which kind of SYT family is expressed and the roles of the expressed SYT in granulosa cells and in cumulus cells during ovulation. Therefore, we sought to determine whether induction of *Syt* mRNA and binding of SYT1 to SNAP25 in granulosa and cumulus cells are involved in exocytosis during ovulation. In this study, we analyzed the kinetic changes of SYT family expressions in granulosa cells and in cumulus cells during ovulation. The binding of SYT1 to SNAP25 was detected by immunoprecipitation study.

Materials and Methods

Materials

Equine chorionic gonadotropin, eCG and hCG were purchased from Asuka Seiyaku (Tokyo, Japan). Oligonucleotide poly-(dT) was purchased from GE Healthcare (Buckinghamshire, UK), and AMV reverse transcriptase and Taq polymerase were purchased from Promega (Madison, WI, USA). Routine chemicals and reagents were obtained from Nakarai Chemical Co. (Osaka, Japan) or Sigma Chemical Co. (Sigma; St. Louis, MO, USA).

Animals

Immature female C57BL/6 mice were obtained from Clea Japan (Tokyo, Japan). At 23 days of age, female mice were injected intraperitoneally (IP) with 4 IU of eCG to stimulate follicular growth followed 48 hr later by injection of 5 IU hCG to stimulate ovulation and luteinization [16, 17]. The animals were housed under a 16-hour light/8-hour dark schedule in the Experiment Animal Center at Hiroshima University and were provided food and water ad libitum. They were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals, as approved by the Animal Care and Use Committee at Hiroshima University.

RT-PCR analyses

Total RNA was obtained from granulosa cells recovered from eCG-primed ovary or brain tissue using an RNAeasy mini kit (Qiagen Sciences, Germantown, MD, USA) according to the manufacturer's instructions. Briefly, total RNA was reverse transcribed using 500 ng poly-dT and 0.25 U avian myeloblastosis virus-reverse transcriptase at 42°C for 75 min and 95°C for 5 min. For amplification of the cDNA products, specific primers pairs were selected and analyzed as indicated in Table 1. The amplified products were analyzed by 2% agarose gel electrophoresis.

Real-time PCR analyses

The granulosa cells were collected at 0 (48 hr post-eCG), 4 and 8 hr post-hCG. The cDNAs produced by the above method were also used for real-time PCR analysis. The cDNA and primers were added to a 15 μl total reaction volume containing Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). PCR reactions were then performed using an iCycler thermocycler (Bio-Rad, Hercules, CA, USA). The PCR conditions were set to the following parameters: 10 min at 95°C, 45 cycles each of 15 sec at 95°C and 1 min at 62 or 64°C. Specific primer pairs were selected and analyzed as indicated in Table 1.

Western blot analyses

Protein samples from granulosa cells that were collected at 0 (48 hr post-eCG), 4, 8 and 16 hr post-hCG were prepared by homogenization in whole cell extract buffer and then diluted with the same volume of 2X SDS sample buffer [3]. Extracts (20 μg protein) were resolved by SDS polyacrylamide gel (10%)

Table 1. List of primers employed for RT-PCR and the expected size

	Primer sequence	Annealing temperature	Product Size (bp)
Syt1	F:5'- CTGCCATTCCCTCGTACTGC-3' R:5'- ACAGGAGGCCACACTTTCCA-3'	64	362
Syt2	F:5'-TCTTGCCTCGTCCCTCGTC-3' R:5'-CCACCCAAGCTCCCTTCTT-3'	64	223
Syt3	F:5'- CTCATCTCCTCGAAGCCATAATGT-3' R:5'-CTTGGACAACAGCAAAGAGACAGA-3'	64	255
Syt4	F:5'-CTTGTGCCACTCCCAATTGCT-3' R:5'-TTCGGCTGGACCTAAGTCATACTC-3'	60	155
Syt5	F:5'- GTGGCTCCCAAAGAGGAGCA -3' R:5'- CTGGTCACAGGGCACCTCAA -3	60	264
Syt6	F:5'-TCCCAGAAGTGGGCTCCAAA-3' R:5'-ACCGAGGCTGGTGGTGCTAA-3'	66	266
Syt7	F:5'- AACCCCTCTGCCAACTCCAT-3' R:5'-GCGGCTGAGCTTGTCTTTGT-3'	66	261
Snap23	F:5'-CAACCGAGCCGGATTACAAA-3' R:5'- GGCATTCCTCCTCTGCTCCT-3'	64	387
Snap25	F:5'-GAGATGCAGAGGAGGGCTGAC-3' R:5'- GCTGGCCACTACTCCATCCTG-3'	62	309
L19	F:5'-CTGAAGGTCAAAGGGAATGTG-3' R:5'- GGACACAGTCTTGATGATCTC-3'	60	196

electrophoresis and transferred to PVDF membranes (GE Healthcare). The membranes were blocked in Tris-buffered saline and Tween 20 (TBST; 10mM Tris (pH7.5), 150 mM NaCl and 0.05% Tween 20) containing 5% nonfat Carnation instant milk (Nestle Co., Solon, OH, USA). Blots were incubated primary antibody at 1:1000 dilution of anti-Synaptotagmin 1 monoclonal antibody (R&D Systems, Minneapolis, MN, USA) or a 1:5000 dilution of anti-β-Actin antibody (AC74, Sigma) overnight at 4°C. After washing in TBST, Enhanced chemiluminescence (ECL) detection was performed using an ECL detection system according the manufacture's specifications (GE Healthcare) and by appropriate exposure of the blots to FujiFilm X-ray film (FujiFilm Medical Corp, Tokyo, Japan).

Immunofluorescence

Ovaries were embedded in O.C.T. compound (Sakura Finetek USA Inc.) and stored at –70°C before preparation of 5-micron sections, which were fixed overnight in PBS-buffered 4% paraformaldehyde at 4°C. The sections were then sequentially probed with primary anti-Synaptotagmin 1 antibody and secondary Cy3-conjugated goat anti-mouse IgG antibody (Sigma). Slides were mounted using VectaShield with DAPI (Vector Laboratories, Burlingame, CA, USA).

Immunoprecipitation

Protein samples (100 μg) of granulosa cells were incubated with either anti-Synaptotagmin 1 mouse monoclonal antibody diluted at 1:50 or IgG purified from normal mouse serum (Sigma) at 4°C overnight. The immune complex was then precipitated with Protein A sepharose beads (Sigma), and the precipitate was used for Western blotting as described above.

Statistics

Statistical analyses were carried out for all data from three or four replicates for comparisons by one-way ANOVA followed by Duncan's multiple-range test (Statview; Abacus Concepts, Inc., Berkeley, CA, USA).

Results

The molecules of SNARE complex are abundantly expressed in neuronal and neuroendocrine cells [18]. To detect which types of *Syt* family members are expressed in granulosa cells, we performed a RT-PCR study using both brain tissue as a positive control and granulosa cells. The results show in Fig. 1 that *Syt1*, *Syt2*, *Syt3*, *Syt4*, *Syt6* and *Syt7* mRNA were expressed in both brain tissue and granulosa cells. The *Syt5* mRNA expression was also detected in brain tissue, but

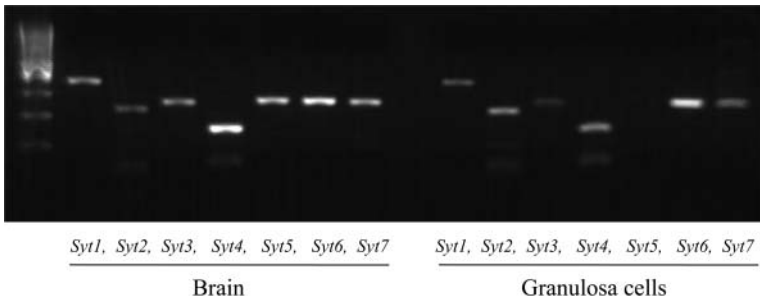

Fig. 1. The expressions of Syt family members in brain tissue as a positive control and in granulosa cells.
The expression of *Syt1*, *Syt2*, *Syt3*, *Syt4*, *Syt5*, *Syt6* and *Syt7* mRNA in brain tissue and granulosa cells recovered from an eCG-primed mouse ovary was determined by RT-PCR.

no PCR product amplified by *Syt5* primers was observed in granulosa cells.

Since it has been reported that SYT3 and SYT5 do not have a Ca^{2+} binding motif and that they bind to SNAP23 [19], we examined the kinetic changes of *Snap23*, *Snap25*, *Syt1*, *Syt2*, *Syt3*, *Syt4*, *Syt6* and *Syt7* mRNA expression in granulosa cells during ovulation by real-time PCR. The levels of *Snap23*, *Syt3* and *Syt7* mRNA were not dramatically changed by hCG stimulation (Fig. 2). However, hCG injection significantly increased *Syt1*, *Syt4* and *Snap25* gene expressions within 4 hr (Fig. 2). The significant inductions of *Syt2* and *Syt6* gene expressions were also observed at 8 hr after hCG stimulation (Fig. 2).

Western blot analyses using specific antibodies to SYT1 and SNAP25 showed that the levels of both proteins were increased in the granulosa cells collected at 4, 8 and 16 hr post-hCG compared with 0 hr (Fig. 3a). Positive immunofluorescent signals were detected in theca cells using an anti-SYT1 antibody, but these signals were very week in granulosa cells and in cumulus cells, and few cells were stained in preovulatory follicles (before hCG stimulation; Fig. 3b). However, in ovulating follicles (at 8 hr after hCG stimulation), positive signals localized SYT1 to granulosa cells and cumulus cells (Fig. 3b). To determine whether SYT1 binds to SNAP25 in granulosa cells, we collected granulosa cells from the mice at 0, 8 or 16 hr post-hCG injection, and cell lysates were then used for immunoprecipitation study. The SYT1 antibody coimmunoprecipitated SNAP25 in samples collected from 8 and 16 hr post-hCG (Fig. 3c). The binding of SYT1 to SNAP25 was suppressed in the presence of EDTA, suggesting that complex formation is Ca^{2+}-dependent (Fig. 3c).

Discussion

SNAP25 and the SNARE complex have specialized functions in fast-regulated secretion pathways, such as synaptic vesicle exocytosis [18]. Recently, SNAP25 expression has been observed in non-neuronal cells, such as granulosa cells [5, 20], pancreatic beta cells [21], chromaffin cells [22, 23] and sperm [24]. In chromaffin cells, the Ca^{2+}-triggered exocytosis burst responsible for regulated catecholamine secretion requires SNAP25 and associated SNARE proteins [22], which suggests that SNAP25 participates in regulated stimulus driven secretory processes in these non-neuronal cells. The present study showed that *Syt* family members are induced markedly in granulosa cells and cumulus cells of ovulating follicles and that SYT1 directly binds to SNAP25 in a Ca^{2+}-dependent manner. Thus, regulation of exocytosis might be required for these cells to release specific vesicle-contained factors.

Interestingly, the expression levels of *Syt3* and *Syt7* were not affected by hCG stimulation. Both types were also localized on the secreted vesicle and moved the vesicle to the cell membrane via an interaction with SNAP23 even if under low Ca^{2+} conditions [13, 19]. The SNARE complex constituted of SNAP23 and either SYT3 or SYT7 is involved in the basal level (non-regulated type) of granule release in endocrine cells [13]. Although the expression of *Snap25* and *Syt1* was significantly upregulated by hCG, the *Snap23* level was not increased in granulosa cells during ovulation, indicating that the release of secreted factors from granulosa cells and cumulus cells is mainly regulated by the increase in the Ca^{2+} level in the cells.

It is possible that the Ca^{2+}-dependent exocytosis

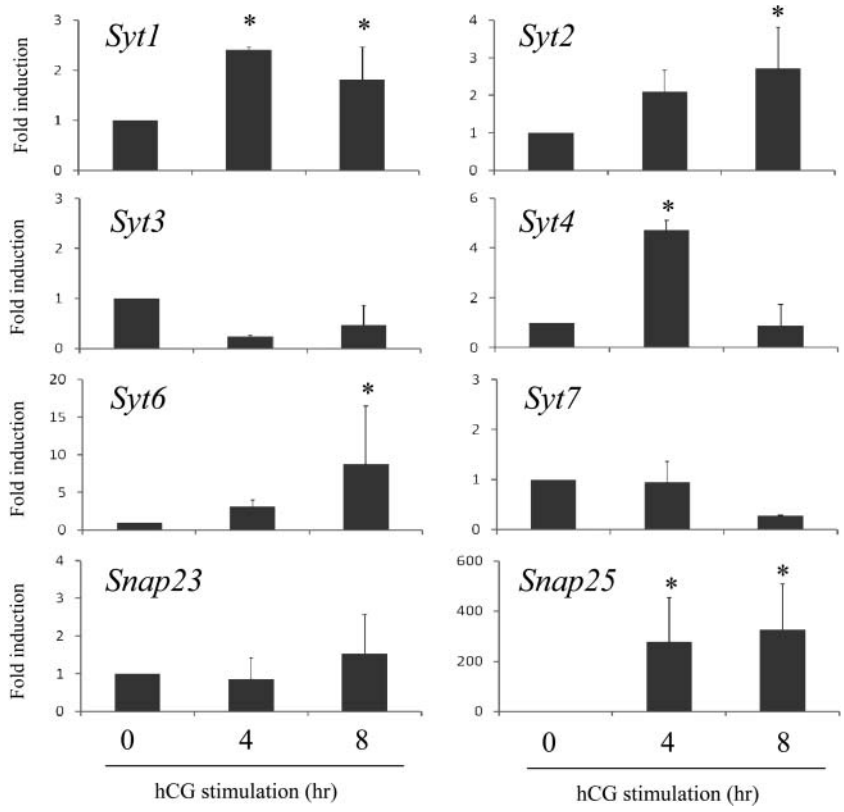

Fig. 2. Kinetic changes in the expression of *Syt* family members (*Syt1*, *Syt2*, *Syt3*, *Syt4*, *Syt6* and *Syt7*), *Snap23* and *Snap25* in granulosa cells during ovulation process.
For reference, the 0 hour value was set as 1, and the data are presented as fold increases. Values are means +/–SEM of three replicates. *Significant differences ($p<0.05$) were observed compared with the value for granulosa cells recovered from mice without hCG injection (0 hr).

system in cumulus cells is involved in fertilization. Our previous study showed that when ovulated mouse cumulus-oocyte complexes were cultured with sperm (*in vitro* fertilization), an initial rapid release of IL6, CCL4 and CCL5 from cumulus cells was observed within 30 min, and these inductions did not require *de-novo* mRNA synthesis [25]. The mechanism by which this rapid release is regulated could involve an exocytosis system because the complex of SNAP25 and SYT1 is formed in cumulus cells in a Ca^{2+}-dependent manner. Sperm-induced chemokine secretion from cumulus cells is regulated by tol-like receptor (TLR) 2 and TLR4 in cumulus cells [4, 25]. In mast cells, it has been also reported that vesicle degranulation and release of cytokines occurs in a TLR2- and TLR4-dependent manner [26] via exocytosis involving SNAP25 localized to the secretory granules [27, 28]. Thus, we believe that the SNAP25-SYT1 associated exocytosis system present in cumulus cells is activated by the TLR pathway during the fertilization process, although the precise mechanisms remain to be elucidated.

Human follicular fluid collected from periovulatory follicles contains IL-1β, IL-6, GM-CSF, KC, MCP-1, MIP-1β and RANTES [29–36]. Importantly, we have shown previously in microarray data and a protein array that cumulus cells express and secrete IL family and chemokine families [3, 5]. For example, IL-6 and its receptor are expressed in granulosa cells after hCG injection, and secretion of this cytokine can mediate, by autocrine or paracrine mechanisms, progesterone production in granulosa cells and hyaluronan accumulation [7]. Although we have previously reported that the secretion levels from granulosa cells were significantly decreased by reduction of the SNAP25 protein level [5], there is no information available about the role of the chemokine family in granulosa cells or

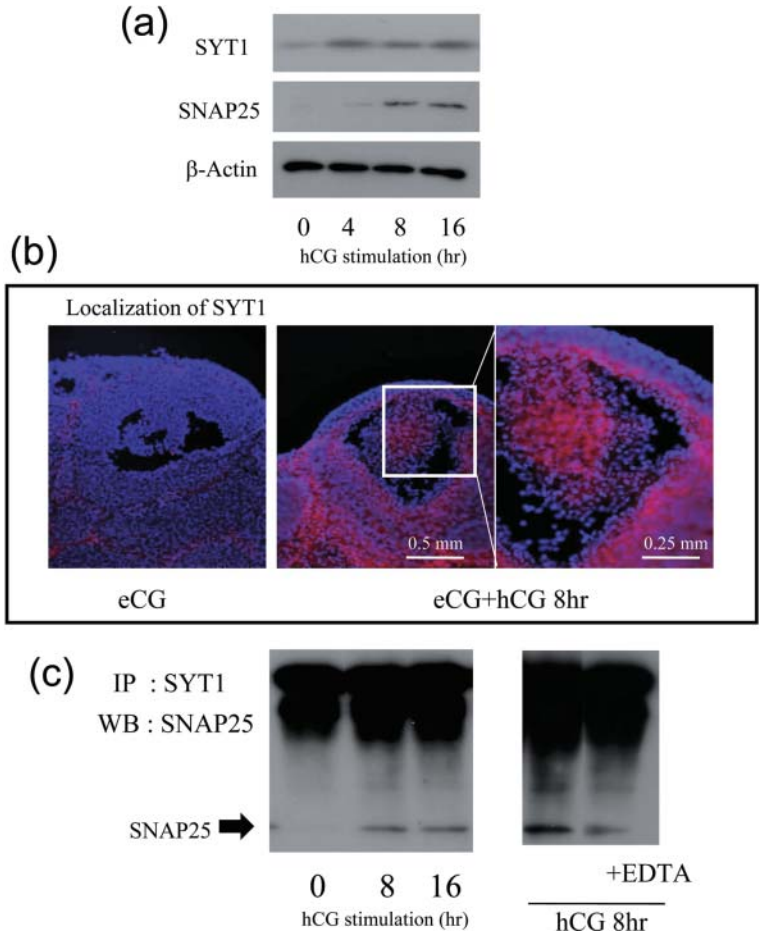

Fig. 3. SYT1 expressed in granulosa cells and cumulous cells of ovulating follicles are functional, and the SYT1 and SNAP25 complexes formed in a Ca^{2+}-dependent manner.
(a) The level of SYT1 and SNAP25 proteins in granulosa cells during ovulation. Granulosa cells were recovered from follicles 4, 8 or 16 hr post-hCG and then used for Western blotting. (b) Localization of SYT1 protein in the ovaries recovered from eCG-primed mice or eCG+hCG primed mice (8 hr after hCG injection). Blue: DAPI staining of nuclei. Red: Cy3 signal localizing anti-SYT1 antibody. (c) The binding of SYT1 to SNAP25 in granulosa cells is upregulated during ovulation. After the granulosa cell lysates were incubated with anti-SYT1 antibody and protein A beads, the precipitated protein was detected by anti-SNAP25 antibody. Some reactions occurred in the presence of 5 mM EDTA.

how to increase Ca^{2+} in granulosa cells to release secreted factors.

CC chemokine family members (CCL2, CCL3 CCL4 and CCL5) are predominantly produced by granulosa cells and cumulus cells at a level of more than 100 pg/ml [5, 25]. Whereas CCL2 stimulates the CCR2 receptor, CCL3 mainly binds to CCR1 and partly to CCR5, and CCL4 selectively activates CCR5 [37]. All of these receptors (CCR1, CCR2, CCR3 and CCR5) are activated by CCL5 [37]. The CCRs are members of the G-protein coupling receptor (GPCR) family that induces phospholipase C to increase Ca^{2+} and PKC activation in cytoplasm [38]. Expression of *Ccr1*, *Ccr2*, *Ccr3* and *Ccr5* mRNAs has been detected in cumulus cells and in granulosa cells from periovulatory follicles by microarray analysis and RT-PCR study (data not

shown). The above observations appear to be part of an autocrine pathway of exocytosis system in granulosa cells and cumulus cells because chemokines can enhance exocytosis by activation of GPCR (CCRs) and calcium release to bind SYT1 to SNAP25, although the precise mechanisms remain to be elucidated.

In the present study, after hCG stimulation, the expression levels of *Syt1*, *Syt2*, *Syt4* and *Syt6* were induced markedly in granulosa cells with induction of *Snap25* during ovulation. The levels of SYT1 protein were also increased 4 hr after hCG injection and remained increased until 16 hr post-hCG. Immunofluorescence using an SYT1 specific antibody showed increased staining for SYT1 in granulosa cells and cumulus cells of hCG-primed mice. Binding of SYT1 to SNAP25 in the granulosa cells accumulated from 8–16 hr post-hCG and was induced by Ca^{2+} dependent manner. From these results, we conclude that the interactions of SYT1 and SNAP25 facilitate the exocytosis process in granulosa cells and in cumulus cells to control the release of factors including specific cytokine family members.

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