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Physiology

Annexin A1 is a novel target gene of gonadotropin-releasing hormone in LβT2 gonadotrope cells

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ABSTRACT. Gonadotropin-releasing hormone (GnRH) regulates gonadotropin secretion. We previously demonstrated that the expression of annexin A5 (ANXA5) is stimulated by GnRH in gonadotropes and has a significant role in gonadotropin secretion. It is therefore of interest to know whether other members of the ANXA family, which consists of twelve structurally related members, are also regulated by GnRH. Therefore, the expression of all annexins was examined in LβT2 gonadotrope cells. *ANXA4*, *A5*, *A6*, *A7* and *A11* were detected in LβT2 cells. The expression of *ANXA5* and *A1* mRNA was stimulated by a GnRH agonist. An increase in ANXA1 protein by this agonist was demonstrated by western blotting. Immunohistochemistry showed that ANXA1 was present in the nucleus and to a lesser extent in the cytoplasm of some rat pituitary cells. The GnRH agonist induced translocation of ANXA1 to the periphery of LβT2 cells. The presence of ANXA1 in gonadotropes and its increase upon GnRH agonist treatment were confirmed in a primary pituitary cell culture. *ANXA1* expression was also demonstrated in the ovary, the testis, the thyroid gland and the pancreas in a different manner to that of *ANXA5*. These data suggest that *ANXA1* is a novel GnRH target gene in gonadotropes. *ANXA1* also may be a target of local GnRH in peripheral tissues and may have a different role than that of *ANXA5*.

KEY WORDS: annexin A1, annexin A5, cell biology, GnRH, gonadotrope

Gonadotropin-Releasing Hormone (GnRH) is a hypothalamic decapeptide hormone that is delivered to the anterior pituitary gland through the pituitary portal system [18]. The GnRH receptor has been detected in the pituitary gland and also in other peripheral tissues, suggesting that it has a wide variety of functions other than regulating gonadotropin secretion [10]. GnRH affects the expression of more than 200 genes in gonadotropes [11]. We have previously found that annexin A5 (ANXA5) is expressed in gonadotropes and that GnRH directly stimulates ANXA5 expression [12, 15–17].

Annexins constitute a family of structurally similar proteins that exhibit the common characteristic of calcium-dependent phospholipid binding [4, 7]. Annexins are widely detected in eukaryotes. In vertebrates, twelve annexins were reported as annexin A, and these were named as ANXA1 to A13, while A12 was unassigned [20]. Annexins consist of a conserved C-terminal core domain, four (eight for ANXA6) repeats of an approximately 60 amino acid sequence, and a variable N-terminus [4].

Since we detected the expression of ANXA5 in the pituitary gland [15], we showed that ANXA5 is expressed in pituitary gonadotropes and that GnRH augments *ANXA5* mRNA expression [12, 17]. We therefore hypothesized that ANXA5 would play a role in the process of GnRH action in gonadotropes. Indeed, downregulation of ANXA5 expression results in decreased gonadotropin secretion [12]. Interestingly, this relationship between GnRH and ANXA5 also takes place in peripheral tissues, e.g. in the ovary, testis and mammary tissues [14, 24, 32]. GnRH also stimulates ANXA5 expression in these tissues and GnRH was shown to simultaneously induce apoptosis and ANXA5 expression [14, 24]. To date, ANXA5 is the only annexin whose expression has been reported to be under the control of peptide or protein hormones. As the physiological role of many of the annexins is still unclear, it is necessary to examine all annexins in gonadotropes to determine whether their expression is related to GnRH or not.

We found that *ANXA1* is a novel target gene of GnRH in gonadotropes. The expression of *ANXA1* was dramatically stimulated by GnRH, and GnRH changed the localization of ANXA1 in these cells. Specific distribution of ANXA1 was observed in the pituitary gland and also in peripheral tissues.

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Table 1. Primers used for real-time PCR

ANXA1	Forward : 5′- ATGTTGCTGCCTTGCACAAA-3′
	Reverse : 5′- CCAAGGGCTTTCCATTCTCCT-3′
ANXA2	Forward : 5′- CTTCAAGGGAGGCTCTCAGC-3′
	Reverse : 5′- GTAGAATGATCACCCTCCAGGC-3′
ANXA3	Forward : 5′- GCCTCTATCTGGGTTGGACC-3′
	Reverse : 5′- GTCCCAAGTCCTCTGATCGC-3′
ANXA4	Forward : 5′- TTTCTCCGCACCAGAGGAAC-3′
	Reverse : 5′- CAGGGTCTGGGCATCTTCAG-3′
ANXA5	Forward : 5′- CTCTGTTTGGCAGGGACCTT-3′
	Reverse : 5′- GGCATCGTAGAGTCGTGAGG-3′
ANXA6	Forward : 5′- TGGCCACTTCAGAAGGATTCTC-3′
	Reverse : 5′- GTGTCTGCTATTTCCTGGGCA-3′
ANXA7	Forward : 5′- TCTCGTTCAGAATGTCATACCCA-3′
	Reverse : 5′- CGCAGTGGGGAAAGATGACT-3′
ANXA8	Forward : 5′- AGAGGAGCAATGTGCAGAGG-3′
	Reverse : 5′- GTATGGCGGGTACATGAGGG-3′
ANXA9	Forward : 5′- CAGACTAAGGGTTCAGGGGAC-3′
	Reverse : 5′- TCAGAGATGTTCCCAGTGGTC-3′
ANXA10	Forward : 5′- ACCTCAGGACACTTCAGGGAT-3′
	Reverse : 5′- TTCCCACAGCACCATTGCAT-3′
ANXA11	Forward : 5′- GCAGACACTTCTCGGGTTAGG-3′
	Reverse : 5′- CTCATGGCTAGAGCTGGCAC-3′
ANXA13	Forward : 5′- GGGGGAAGAACCCGAACATT-3′
	Reverse : 5′- TCTTTACGCCGCATTAGCCA-3′
RPL19	Forward : 5′- CTGATCAAGGATGGGCTGAT-3′
	Reverse : 5′- TTCAGCTTGTGGATGTGCTC-3′

MATERIALS AND METHODS

LβT2 gonadotrope cells

The gonadotrope-derived cell line LβT2 was a kind gift from professor P. Mellon of the University of California, San Diego. The cells were cultured in Dulbecco's Modified Eagle Medium with low glucose (Invitrogen, Tokyo, Japan) supplemented with 10% fetal bovine serum (Gibco Life Technologies, Grand Island, NY, U.S.A.) and an antibiotic-antimycotic mixture (Gibco Life Technologies). The cells were grown in 75-cm^2 flasks and maintained in an atmosphere of 95% air, 5% CO_2, and 100% humidity at 37°C. The cells were sub-cultured before becoming confluent.

RNA extraction and cDNA synthesis

Total RNA was extracted by the acid-guanidinium thiocyanate-phenol-chloroform method using TRIzol (Ambion, Thermo Fisher Scientific, Yokohama, Japan) according to the manufacturer's instructions. Total RNA samples were dissolved in DNase/RNase-free water (UltraPureTM Distilled Water; Invitrogen Life Technologies, Grand Island, NY, U.S.A.) to a concentration of 500 *ng*/*μl* and were subjected to reverse-transcription using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Thermo Fisher Scientific, Yokohama, Japan) and a Veriti Thermal cycler (Applied Biosystems, Thermo Fisher Scientific) according to the protocol supplied by the manufacturer. Reverse transcription was performed at 25°C for 10 min, 37°C for 120 min and 85°C for 5 sec, followed by immediate cooling. Complimentary DNA (cDNA) samples were stored at −80°C until real time reverse transcription polymerase chain reaction (RT-PCR) analysis.

Real time RT-PCR

cDNA samples were analyzed by quantitative real-time PCR using the StepOnePlus Real-time PCR system (Applied Biosystems, Thermo Fisher Scientific). The THUNDERBIRD SYBR qPCR Master Mix (Toyobo, Tokyo, Japan) was used for the real time PCR reaction mixture according to the protocol described by the manufacturer. The primers used for real time PCR are listed in Table 1. *RPL19* was used for the internal standard and expression level was calculated by the delta delta ct method.

Changes in ANXA1 and ANXA5 expression in LβT2 cells upon GnRH agonist administration

To examine changes in *ANXA1* and *ANXA5* expression in LβT2 cells upon GnRH agonist treatment (Des-Gly10 [Pro9]-GnRH ethylamide; Intervet KK, Tokyo, Japan) administration, LβT2 cells were incubated with 10^{-7} M of a GnRH agonist for 1, 3 or 9 hr.

Total RNA was then extracted and used for measuring ANXA1 and ANXA5 mRNA expression using quantitative RT-PCR.

Western blotting

LβT2 cells were cultured in 35 mm dishes for 48 hr and were then incubated with 10^{-7} M of the GnRH agonist for 3 hr. Total protein of intact or GnRH agonist-stimulated LβT2 cell lysates (20 μg) was separated on a 12% SDS-PAGE gel and the proteins were transferred to 0.45 μm PVDF membrane (Amersham Hybond, GE Healthcare Life Science, Germany). The membrane was blocked with 5% non-fat milk containing 1% Tween 20 in PBS for 1 hr at room temperature with gentle shaking. ANXA1 was immunodetected with a 1:10,000 dilution of a polyclonal anti-ANXA1 antibody (Thermo Fisher Scientific) in blocking solution at 4°C overnight. Anti-rabbit IgG-conjugated horse radish peroxidase (ICN Immuno-biological laboratories, Minneapolis, MN) at a 1:50,000 dilution was used as the secondary antibody to detect ANXA1 immunoreactivity with ECL Western Blotting Detection Reagents (GE Healthcare Japan, Tokyo, Japan). Chemiluminescence was detected using the ImageQuant LAS 4000 series. The membrane was directly re-probed with a 1:5,000 dilution of a monoclonal anti-β–actin antibody (Santa Cruz, CA, U.S.A.) as an internal control.

Immunofluorescence analysis of ANXA1 in LβT2 cells and primary pituitary cells

LβT2 cells (100,000 cells) were seeded on poly-L-lysine-coated coverslips in 35 mm dishes. One day after plating, the medium was replaced. These LβT2 cells were then incubated with or without 10^{-9} M of the GnRH agonist for 3 hr. The LβT2 cells were fixed with 4% PFA and rinsed with cold acetone. Immunofluorescence was performed using a 1:1,000 dilution of a polyclonal rabbit anti-ANXA1 antibody (Thermo Fisher Scientific) and bound antibody was detected using goat anti-rabbit IgG-Alexa Fluor 488 (Thermo Fisher Scientific) as the secondary antibody. Specimens were mounted with Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI, VECTOR Laboratories, Burlingame, CA, U.S.A.). ANXA5 immunofluorescence was observed using a confocal laser microscope and actin was stained with phalloidin (Zeiss LSM710, Tokyo, Japan).

Immunocytochemistry was also performed with primary pituitary cells. Primary cultures of rat anterior pituitary cells were prepared as reported previously [12]. Briefly, anterior pituitary glands from estrous cycling rats, regardless of their stages, were collected shortly after decapitation and dispersed with 0.25% trypsin. Primary cells were seeded on poly-L-lysine coated coverslips in 35 mm dishes and maintained in modified Eagle Medium with low glucose supplemented with 10% fetal bovine serum (Gibco Life Technologies) and an antibiotic-antimycotic mixture (Gibco Life Technologies). Double-immunocytochemistry was also performed with rabbit anti-ANXA1 serum and guinea pig anti LHβ serum after GnRH agonist (10^{-9} M) treatment for 48 hr. Control specimens cultured without the GnRH agonist were also prepared. Second antibodies were anti-rabbit IgG-Alexa fluor 488 and goat anti-guinea pig IgG Alexa fluor 568.

Animals

Adult Wistar Imamichi rats that were bred in our laboratory were used in the study. They are also commercially available from the Institute for Animal Reproduction (Ibaraki, Japan). The rats were maintained in light- (5:00–19:00 hr) and temperature- (23 ± 3°C) controlled room. They were fed with laboratory chow and tap water *ad libitum*. All procedures were performed according to the guidelines for animal treatment at Kitasato University after receiving approval from the president of Kitasato University and Institutional Animal Care and Use Committee (Approval no. 15-029).

Immunohistochemistry of ANXA1 and ANXA5

The anterior pituitary gland, ovary, testis, adrenal gland and pancreas were harvested from male and female rats. Tissues were fixed with 4% paraformaldehyde overnight. After washing the tissues with PBS, dehydration was performed as per the standard procedure. Paraffin blocks were used for making tissue sections of 2 μM thickness. De-paraffinization was performed using xylene and ethanol series. Endogenous peroxidase activity was eliminated by pretreating the tissue sections with 1% hydrogen peroxide in methanol for 20 min. Tissue sections were then blocked with 2.5% normal horse serum (ImmPress Reagent, Vector Laboratories, Inc.) for 1 hr at room temperature. Incubation with the primary rabbit anti-ANXA1 (1:1,000) or anti-ANXA5 (1:5,000) antibody (homemade, against recombinant rat ANXA5) [13] was performed overnight at 4°C in a humidified chamber. Incubation with the second antibody, peroxidase labeled anti-rabbit IgG antibody (ImmPress Reagent, Vector Laboratories, Inc.) was performed for 2 hr at room temperature. Immunoreactions were visualized with DAB. The slides were counterstained with hematoxylin.

Statistical analysis

Mean values were compared using Student's *t* test; $P<0.05$ was considered significant.

RESULTS

Expression of annexin mRNA in LβT2 cells

The mRNA expression of *ANXA1* to *A11* and *A13* in intact LβT2 gonadotrope cells was examined using real time RT-PCR with specific primers for each annexin. *ANXA4*, *A5*, *A6*, *A7* and *A11* mRNA were detected (Fig. 1). As amplification efficacy may vary among each primer set, direct quantitative comparison between annexins is not possible. However, even though the expression rate varied among primers used, *ANXA5* appeared to be a prominent annexin in LβT2 cells, and besides *ANXA5*, multiple annexins were expressed in intact LβT2 gonadotropes.

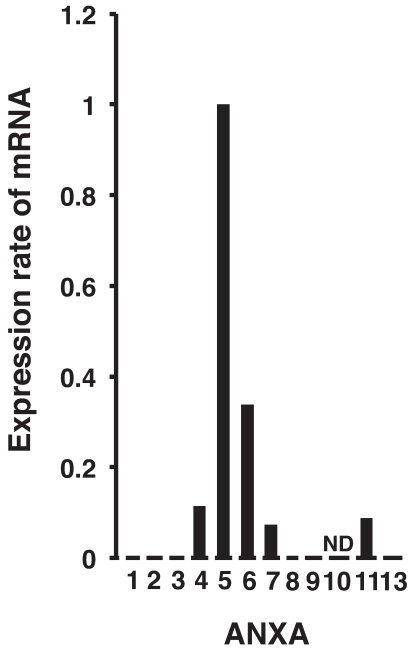

Fig. 1. Rate of annexin mRNA expression in intact LβT2 cells. RNA was obtained from LβT2 cells and was used to determine the mRNA expression of each annexin gene using real time RT-PCR with specific primers. ND: not detectable. Each annexin expression rate is expressed as a ratio to the level of *ANXA5* mRNA.

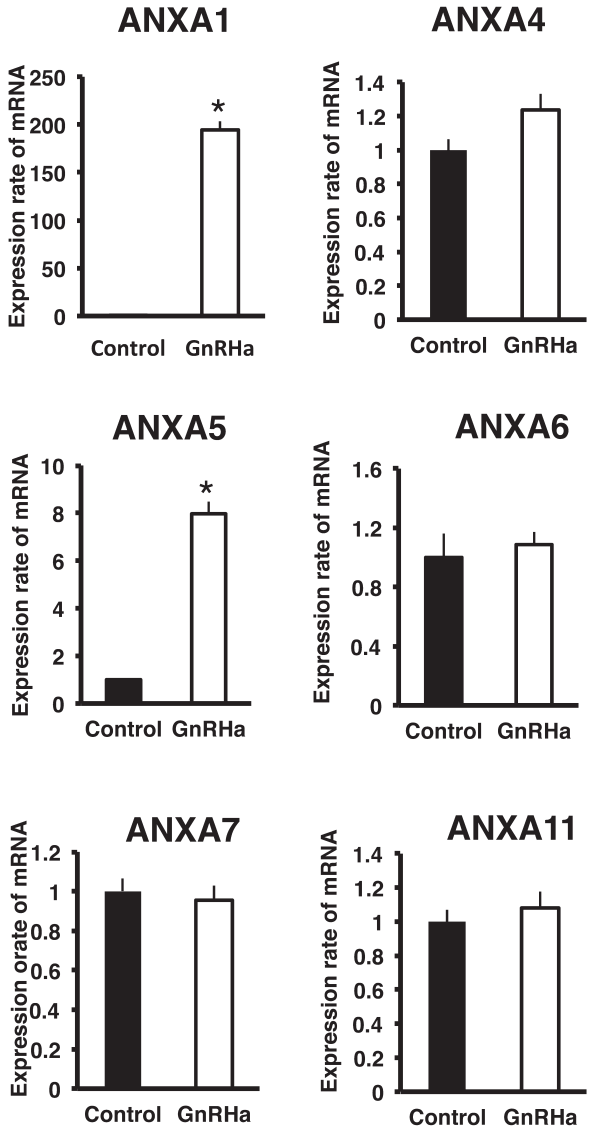

Fig. 2. Effects of GnRH agonist treatment on annexin mRNA expression. The mRNA expression of *ANXA1*, *A4*, *A5*, *A6*, *A7* and *A11* in LβT2 cells was examined using real time RT-PCR after 12 hr of incubation with 10^{-7} M of the GnRH agonist (GnRHa). Asterisks indicate a significant difference from the respective control value, $P<0.05$.

The effect of a GnRH agonist on annexin mRNA expression

We previously found that the expression of *ANXA1* mRNA was increased in the pituitary gland of ovariectomized mice (data not shown). This result suggests that *ANXA1* expression is augmented by GnRH. Therefore, in the present study, the effect of a GnRH agonist on expression of *A1*, *A4*, *A5*, *A6*, *A7* and *A11* mRNA was analyzed. Incubating LβT2 cells with a GnRH agonist for 12 hr significantly augmented the expression of *ANXA1* and *A5* mRNA. At this time point, the *ANXA1* mRNA expression rate had dramatically increased to 194 fold that at 0 hr, while the expression of *ANXA5* mRNA had increased to 7.9 fold that at 0 hr. The rate of *ANXA4*, *A6*, *A7* and *A11* mRNA expression was not changed by treating with the GnRH agonist (Fig. 2).

Time course study of the expression rate of ANXA1 and A5 in LβT2 cells after GnRH agonist administration

To examine changes in the expression rate of *ANXA1* and *A5* induced by the GnRH agonist over time, LβT2 cells were incubated with the GnRH agonist for 0, 1, 3 or 9 hr. After 9 hr, GnRH agonist treatment significantly increased the expression rate of *ANXA5* mRNA to 4.95-fold that at 0 hr. The expression rate of *ANXA1* mRNA was dramatically augmented by the GnRH agonist at 3 hr (12.97-fold increase) and 9 hr (25.59-fold increase) of treatment compared to that at 0 hr (Fig. 3). The mRNA of both annexins was slowly increased over time by treatment with the GnRH agonist. The increase in *ANXA1* mRNA was much higher than that in *ANXA5* mRNA observed at the beginning of the incubation.

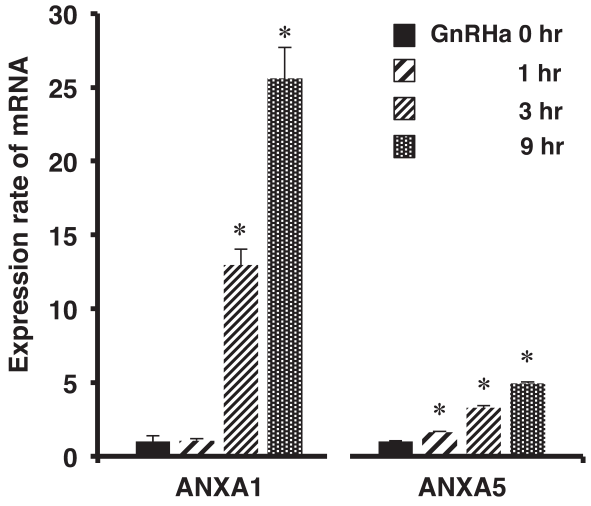

Fig. 3. Changes in the rate of *ANXA1* and *A5* mRNA expression over time in LβT2 cells after GnRH agonist stimulation. The GnRH agonist (10^{-7} M) was administered to LβT2 cells and the cells were harvested after 0, 1, 3 and 9 hr of incubation. *ANXA1* and *A5* mRNA was then analyzed using real time RT-PCR. Asterisks indicate a significant difference from the 0 hr value of each group, $P<0.01$.

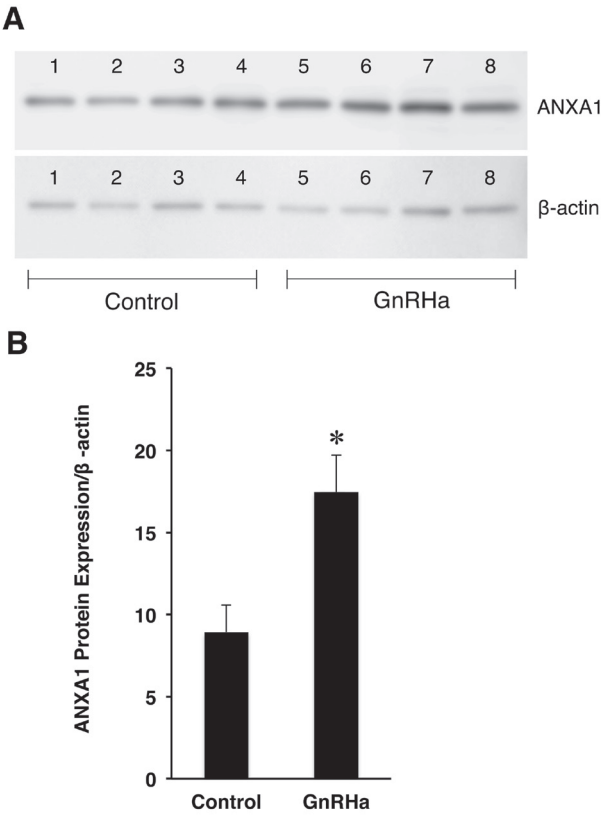

Fig. 4. ANXA1 protein expression after GnRH agonist stimulation. ANXA1 protein expression was estimated by western blotting after GnRH agonist administration. LβT2 cells were challenged with 10^{-7} M of the GnRH agonist for 3 hr and cell lysates were analyzed using SDS-PAGE and western blotting with an anti-ANXA1 antibody. (A) Immunostaining image after western blotting. Lanes 1–4 are control incubations and lanes 5–8 are GnRH agonist-treated samples. β-actin was used as an internal control. Images were cropped and the original membranes are shown in Supplementary figure. (B) The intensity of each band was measured by densitometry. Intensities were normalized with that of the internal control (β-actin). Values are the mean of four samples with the standard error. An asterisk indicates a significant difference, $P<0.05$.

Increase in ANXA1 protein expression by GnRH agonist treatment

To test whether the translation of ANXA1 is also augmented by treating with the GnRH agonist, ANXA1 protein expression was examined by western blotting. The intensity of the ANXA1 protein band was significantly increased in LβT2 cells after GnRH agonist administration for 3 hr compared to that at 0 hr. The intensity of each band was analyzed and normalized to β-actin as an internal control (Fig. 4).

Immunohistochemistry for ANXA1 and A5 expression in pituitary tissues

The distribution of ANXA1 and A5 in rat pituitary tissues was compared using immunohistochemistry. Only a small number of pituitary cells were strongly ANXA1-positive, and in those cells, ANXA1 expression was observed mainly within the nucleus. On the other hand, ANXA5 was present in the majority of the pituitary cells and was localized on the plasma membranes and the nuclear envelope with some intra-nuclear staining (Fig. 5A).

Intracellular localization of ANXA1 in LβT2 cells and pituitary cells

Changes in the distribution of ANXA1 in LβT2 cells over time upon incubation with the GnRH antagonist were observed by immunofluorescent analysis of LβT2 cells. Intra-nuclear and cytoplasmic distribution of ANXA1 was observed at time 0. Interestingly, some puncta of strong ANXA1 immunoreactivity in the cytoplasm were also observed at time 0, which are indicated with red arrows in Fig. 5B. ANXA1 had spread to the periphery of cells after 30 min of incubation, as shown by the yellow arrows in Fig. 5B and 5C. The accumulation of ANXA1 at the periphery and ANXA1 nuclear localization had disappeared by 180 min of GnRH antagonist incubation. Primary cultures of anterior pituitary cells were prepared to demonstrate the co-localization of

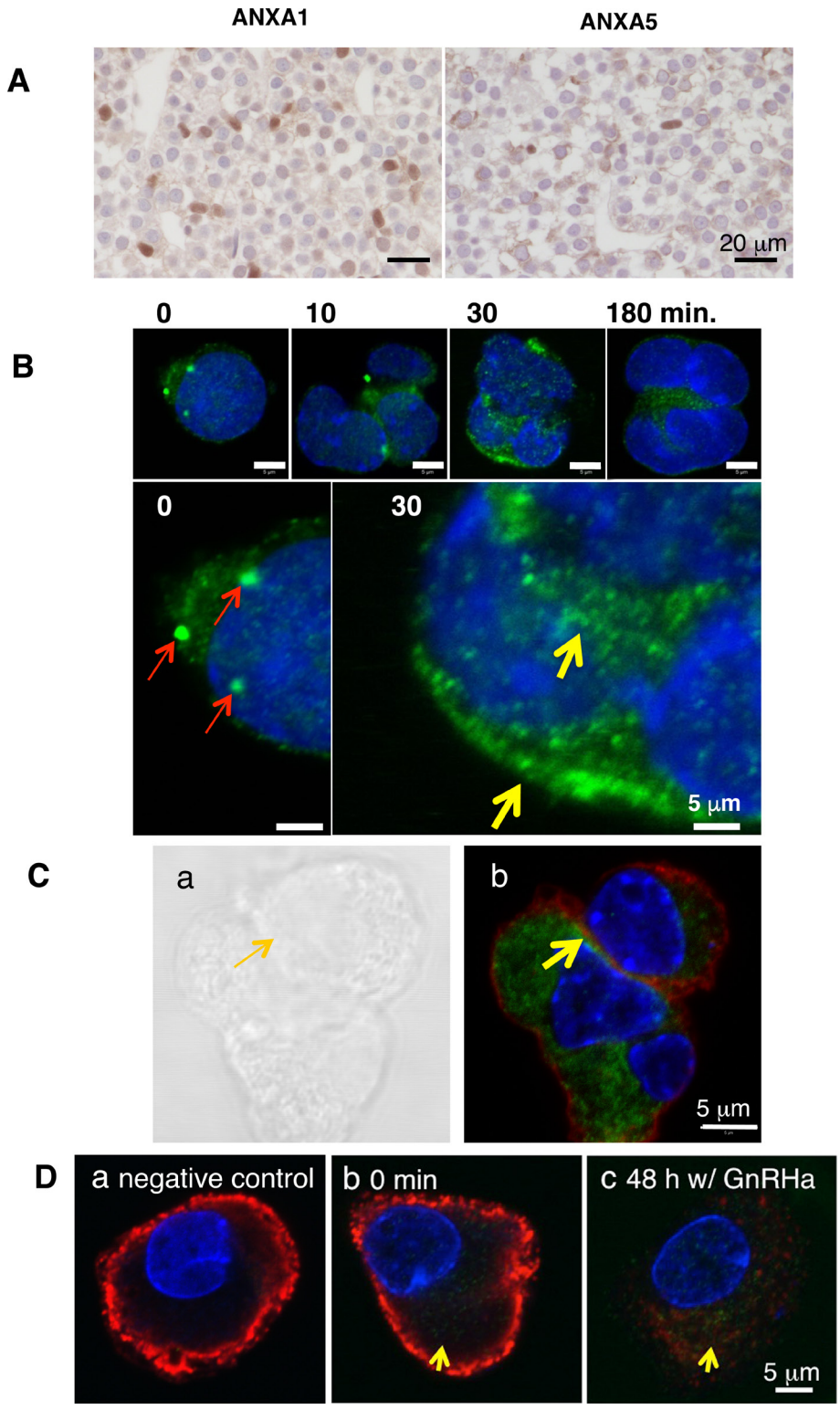

Fig. 5. Immunohistochemical and immunofluorescence analysis of annexin expression. A: ANXA1 and A5 expression in pituitary tissues of female rats was analyzed using immunohistochemistry. B: LβT2 cells were grown on a coverslip and challenged with the GnRH agonist (10^{-9} M) at time 0, then ANXA1 expression over time was analyzed with an anti-ANXA1 antibody using immunofluorescence. ANXA1 is shown with green fluorescence. DAPI was used to stain the nucleus (blue). Red arrows indicate accumulation of ANXA1 in immunoreactive puncta. Yellow arrows indicate ANXA1 accumulation at the periphery of cells. C: ANXA1 accumulation (yellow arrow) to the periphery of LβT2 cells (b) shown with a differential interference contrast (dic) image (a). Actin is shown in red from phalloidin staining. ANXA1 is shown with green fluorescence. DAPI was used to stain the nucleus (blue). D: The coexistence of ANXA1 and the LHβ subunit was shown with double staining immunohistochemistry. Cells were stained with anti-LHβ (red) and anti-ANXA1 (green). Nuclei were stained with DAPI (blue). a: negative control for ANXA1. The cells were stained without anti-ANXA1. b: Cells were stained before exposure to the GnRH agonist. c: The cell after 48 hr of incubation with GnRH agonist (10^{-9} M) was double stained. Yellow arrow indicates ANXA1 in the cytoplasm.

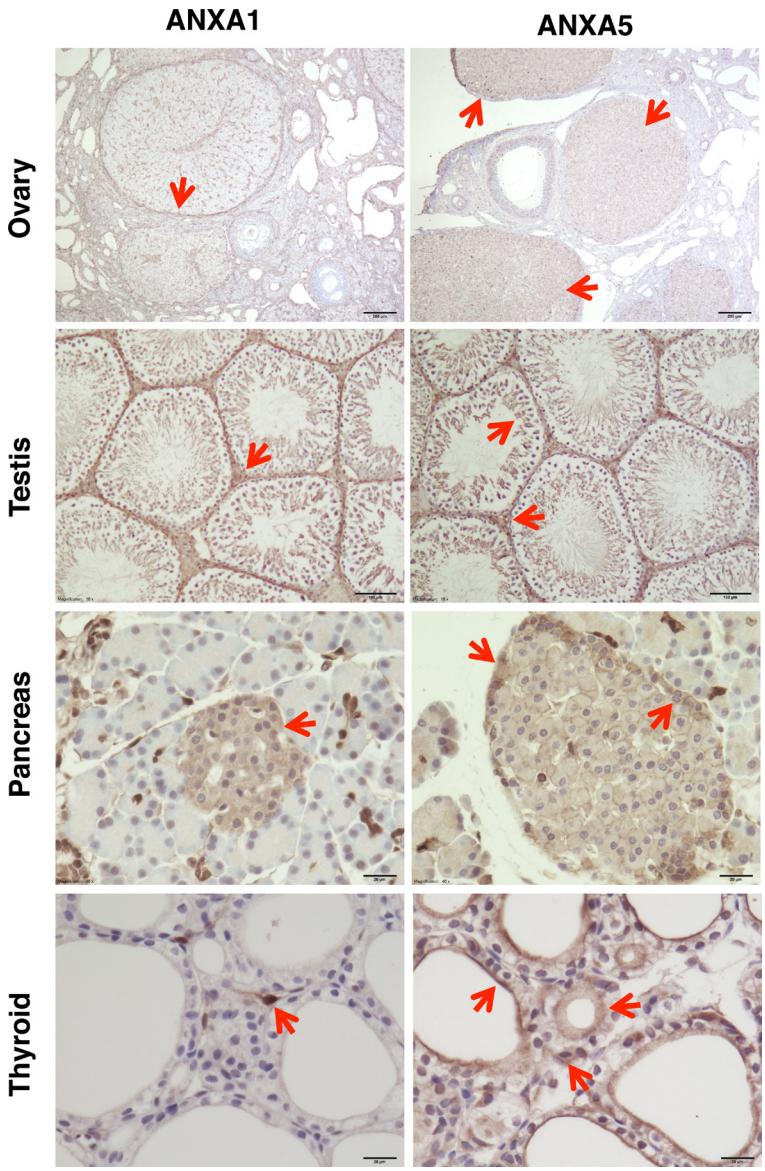

Fig. 6. Immunohistochemical analysis of ANXA1 and A5 expression in endocrine organs. ANXA1 and A5 expression in rat ovary, testis, pancreas and thyroid tissues was analyzed using immunohistochemistry. Red arrows represent a typical positive reaction in each tissue.

the LHβ subunit and ANXA1. Cells were incubated with the GnRH agonist for 48 hr. While there was no green signal in negative staining (Fig. 5D-a), slight staining with ANXA1 was seen in the cytoplasm of pre-incubated cells (Fig. 5D-b). The cytoplasmic ANXA1 was increased and LHβ was decreased after 48 hr of incubation with GnRH agonist (Fig. 5D-c).

Immunohistochemistry for ANXA1 in other endocrine organs

The expression of ANXA1 and A5 in the rat ovary, testis, pancreas and thyroid gland was also examined (Fig. 6). Cross-reactivity of antibodies to ANXA1 and A5 would be negligible since both antibodies detected only one protein band in western blot analysis. In the ovary, granulosa cells appeared to be negative for ANXA1. Theca cells and small luteal cells were the main ANXA1-positive cells in the ovary. Granulosa cells were also negative for ANXA5, which was expressed in the corpus luteum. However, the staining patterns of ANXA1 and A5 differed; ANXA5 staining was more diffuse than ANXA1 staining. Large steroid-producing cells appeared to be negative for ANXA1. In the testis, the expression pattern of ANXA1 and A5 was similar. Leydig cells and the nuclei of sperm cells were positive for both ANXA1 and A5 (Fig. 6). In the pancreas, an immunopositive reaction for ANXA1 and A1 was seen in some parts of the exocrine gland. On the other hand, the pancreatic endocrine tissue, the islets of Langerhans, was broadly stained with ANXA1. ANXA5 was also expressed in these islets and in the plasma membranes of large round cells, and the cytoplasm of islet peripheral cells stained positively for ANXA5. Finally, the expression pattern of these proteins in the thyroid gland was compared. Only large interstitial cells were positive for ANXA1, while ANXA5 was detected in follicular epithelial cells, especially on the inner surface of the follicles.

DISCUSSION

We demonstrated here for the first time that the expression of *ANXA1* mRNA is stimulated by GnRH in gonadotropes. Treating with a GnRH agonist dramatically augmented the expression of *ANXA1* in the pituitary gonadotrope cell line, LβT2. The extent of *ANXA1* augmentation was markedly higher than that of *ANXA5*. Expression of other annexins was not affected by the GnRH agonist. Thus, of the 12 mammalian annexins, *ANXA5* and *A1* were the only GnRH-responsive annexins. As we have already reported that ANXA5 plays a significant role in the GnRH functions of gonadotropin secretion and apoptosis [12, 14, 24], the present data suggest an important, but as yet undefined, role for ANXA1 in GnRH function.

The suppressive activity of ANXA1 on adrenocorticotropic hormone (ACTH) release by the pituitary gland in response to cortisol has been well-studied [2, 27]. Negative feedback of cortisol on ACTH release is mediated by ANXA1 [27]. ANXA1 was also shown to be involved in thyrotropin-releasing hormone (TSH) and prolactin secretion by the same research group [26, 28, 29]. Folliculostellate cells contain ANXA1, and paracrine regulation of ANXA1 by folliculostellate cells in anterior pituitary tissues has been observed [30]. ANXA1 was found to function primarily as a mediator of the anti-inflammatory action of cortisol [6]. Cortisol suppresses phospholipase A_2 by means of ANXA1 [3]. Despite these studies, no report has yet examined ANXA1 in gonadotrope cells. In an ANXA1 knockout mouse ($ANXA1^{-/-}$), aberrant inflammation and resistance to glucocorticoids were reported [8, 25]. The relationship between GnRH and ANXA1 that was indicated in the present study suggests that $ANXA1^{-/-}$ mice would exhibit reproductive failure; however, to date there has been no description regarding gonadotropin secretion or any reproductive complications in $ANXA1^{-/-}$ mice. GnRH stimulates the synthesis of ANXA5, and ANXA5 is involved in gonadotropin secretion [12, 16]. Although we previously found a significant stimulatory effect of ANXA5 on gonadotropin secretion [12], we confirmed the existence of an intact estrous cycle and ovulation in $ANXA5^{-/-}$ mice [31]. Further studies are therefore necessary to clarify the existence of a compensatory mechanism for the depletion of ANXA1 and/or A5. Analysis of the phenotype of ANXA1 and A5 double-knockout mice would also be interesting in this respect.

The expression of ANXA1 and A5 in pituitary tissue was similar but not identical. There were fewer ANXA1-positive cells than ANXA5-positive cells in anterior pituitary tissues. Strong expression of ANXA1 in the nucleus was noted. Neither of these annexins were present in all types of pituitary cells, but ANXA5 seems to be expressed in more pituitary cell types than ANXA1. While ANXA1 exists mainly within the nucleus, ANXA5 is seen on plasma membranes and in the nuclear envelope. These differences in ANXA1 and ANXA5 cellular localization in the pituitary gland suggest that they exhibit distinct mechanisms of action when present in the same cell species. ANXA1 was present in the primary culture of pituitary cells at a very low intensity in the gonadotropes, and its presence was increased after GnRH agonist stimulation. The distribution was different from that observed in pituitary tissues. Immunoreactivity was seen in the cytoplasm of gonadotropes *in vitro*. The expression and distribution of ANXA1 were changed based on the culture condition.

Since we wanted to know whether ANXA1 and A5 play different roles downstream of the GnRH receptor, the expression of ANXA1 and A5 in some peripheral endocrine tissues was compared. The expression pattern of each annexin was found to be quite different. It is interesting that their expression was seemingly cell type- and cell compartment-specific. Although a careful comparison of their distribution is necessary, these data suggested that ANXA1 and A5 shared functions under the control of the GnRH receptor in these organs. ANXA5 inhibits the phosphorylation of ANXA1 by protein kinase C [23]. Although most annexins have been shown to be phosphorylated [9], a phosphorylation site has not been demonstrated in the ANXA5 molecule. These reports and the present data regarding their expression patterns suggest an interrelationship between these two annexins within a cell.

GnRH is a phylogenetically very old peptide hormone [5]. GnRH has been reported even for protochordates that have neither a hypothalamus nor a pituitary gland [18, 19, 22]. Two GnRH molecules have been reported in mammals, GnRH I and II. GnRH I is synthesized in the medial preoptic area of the hypothalamus and is transported to the anterior pituitary gland through the hypophyseal portal system. GnRH I is also synthesized in peripheral tissues. GnRH I is expressed outside of the hypothalamus, for example in the ovary, testis and mammary gland [14, 24, 32]. GnRH II is found mostly in the mid-brain in mammals [21]. We previously found that GnRH augmented ANXA5 expression by treating with a GnRH agonist [14, 24, 32] and have proposed that ANXA5 is a useful biomarker of GnRH action. The present results suggest that ANXA1 would also be a sensitive biomarker of GnRH action.

ANXA1-immunopositive puncta were observed in the cytoplasm of intact cells. This staining may represent the localization of ANXA1 to some organelle or specific functional site in the cell. In the present study, we examined the effect of GnRH on the localization of ANXA1 in gonadotropes and found that a GnRH agonist induced localization of ANXA1 to the cell periphery. It has been reported that ANXA1 is translocated to the outer surface of cells by cortisol in various cell types [1]. It is not known whether ANXA1 was transported outside gonadotropes by GnRH stimulation, similar to the effect of cortisol. However, if this occurred, this phenomenon would likely be related to its function.

The present study clearly demonstrated that ANXA1 is expressed in gonadotrope cells and that its expression is under the control of GnRH. These data suggested that ANXA1 and A5 share GnRH-related functions in gonadotropes and probably also in peripheral tissues.

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