一、 英文摘要： 蔡郁惠

Follicle-stimulating hormone (FSH) binds to its receptor and triggers the cAMP-dependent signaling pathway by activating the Gs protein in Sertoli cells. In the recent study, it is demonstrated that thyroid-stimulating hormone (TSH) receptor was identified to be associated with 10 different G proteins in its intracellular signal transduction pathway. Based on the homology between TSH and FSH receptors, it is asked whether FSH also affects various G protein subfamilies. On the other hand, tissue transglutaminase (tTGase), a G_h protein, was demonstrated to transduce the intracellular signal from α_1-adrenergic receptor to phospholipase C δ-1 (PLC δ-1) and regulate cell proliferation and Ca^{2+} influx in the primary cultured hepatocytes. In this study, the efforts are attempted to investigate whether G_h- PLC δ-1 pathway also plays an important role in FSH-regulated spermatogenesis in Sertoli cells.

Data in this laboratory has shown that FSH elevated intracellular cAMP level in a dose- and time-dependent manner. In addition, intracellular calcium acts as a secondary messenger in the ligand-triggered signaling cascades and regulates the calcium-dependent enzymes, such as tTGase and PLC δ-1. It was subsequently intended to identify whether FSH-induced intracellular calcium oscillation in Sertoli cells was due to Ca^{++} influx or intracellular Ca^{++}-release by controlling the extracellular calcium concentration. The data showed that FSH-induced calcium oscillation revealed two elevation peaks when Sertoli cells were cultured in regular DMEM/ F12 medium. However, the second peak of FSH-induced calcium oscillation was eliminated when Sertoli cells were cultured in calcium-free DMEM/ F12 medium. While the first peak of FSH-induced calcium oscillation was abolished when Sertoli cells pretreated with adenyl cyclase inhibitor.

In this study, the *in situ* tTGase activity analysis revealed that GTPγS decreased the FSH-stimulated tTGase activity in Sertoli cells. Furthermore, the western blotting analysis showed that the FSH-induced increase in PLC δ-1 and G_h protein appeared in the membrane portion of Sertoli cells after FSH treatment. On the other hand, FSH also elevated N-cadherin expression in Sertoli cells by using flow cytomtry analysis. However, further studies are necessary to define the importance of G_h-PLC δ-1 pathway in FSH-regulated physiologic functions of Sertoli cells and FSH-induced signaling pathway involved in Ca^{++}-releasing and influx as well as N-cadherin expression in Sertoli cells.

Keywords: Follicle-stimulating hormone, transglutaminase (tTGase), *in situ* tTGase activity, G proteins, G_h, phospholipase C-δ_1, c-AMP, Sertoli cells.

二、 中文摘要：

促濾泡成長激素(Follicle-stimulating hormone, FSH)與 Sertoli cell 膜上的受器結合後會活化細胞內的 Gs protein，然後觸動一條屬於 cAMP 依賴型的訊號傳遞路徑。近來的研究發現顯示，甲狀腺激素(Thyroid-stimulating hormone, TSH)的接受器在其標的細胞內的訊息傳導中會與超過十種以上不同的 G 蛋白(G-protein)作用。基於此兩種激素之接受器在細胞膜上結構的相似性，因此吾人推測 FSH 可能也會與 Gs protein 以外的 G protein 作用。另一方面，當組織型轉麩胺酶(Tissue transglutaminase, tTGase)與 GTP 結合時，則具有 G 蛋白的功能，其名為 G_h。在肝細胞的實驗中，已發現它可以傳遞來自α1-adrenergic receptor 的信號給磷脂酶 C-δ_1 (phospholipase C δ-1, PLC δ-1)而促進外鈣的引入以及活化一連串與細胞增生有關的調控機制。所以，本計畫之內容主要是探討 G_h 在 FSH 所調控之 spermatogenesis 中是否扮演著重要的訊息傳遞者。

我們的研究結果指出 FSH 可以增加細胞內 cAMP 的含量，而且實驗結果與 FSH 投予的濃度和作用的時間都成正相關。當細胞受到外來的刺激，而觸動細胞內的訊息傳遞時，鈣離子通常扮演 secondary messenger 的角色去調節一些需要鈣來活化的酵素，例如：tTGase 及 PLC δ-1。因此接下來的研究方向就是去探討 FSH 如何去誘導細胞內鈣離子的 oscillation。結果發現在正常的狀態下，以 FSH 處理 Sertoli cells 可發現細胞內鈣的增加會出現雙波狀現象，但是，在移除了細胞外的鈣離子之後，發現只剩下第一個鈣增加的波峰。而事先投予 adenyl cyclase 的抑制劑，發現可降低 FSH 所誘導出之第一個波峰的形成。

在這方面的實驗中，我們利用 *in situ* tTGase activity 的分析法發現 GTPγS 可以降低被 FSH 所活化的 tTGase 活性。而以 GTP-agarose 處理過後的細胞膜萃取物，再以西方墨點法作分析的實驗中，發現在受到 FSH 刺激的 30 及 60 分鐘後，Sertoli cells 中 PLC δ-1 和具有 G protein 功能的 tTGase 蛋白，會於細胞膜的部分開始增多。另外，FSH 也可以促使 N-cadherin 在 Sertoli cell 的表現。然而，接下來的研究必須證明 G_h-PLC δ-1 的路徑在 Sertoli cell 的生理功能上的重要性，以及是否參與了 FSH 在 Sertoli cell 內所觸動的 Ca^{++}-releasing 及 influx，還有 N-cadherin 表現之訊息傳遞路徑。

關鍵詞：促濾泡成長激素， 甲狀腺激素，組織型轉麩胺酶，G 蛋白，*in situ* tTGase activity 分析法，西方墨點法，磷脂酶 C-δ_1.

三、 背景與目的

Follicle-stimulating hormone (FSH) belongs to a family of glycoprotein hormones. FSH and LH (Lutinizing hormone) controls reproductive processes. FSH primarily controls follicular development in the ovary and spermatogenesis in the testis (Richards 1980, McLachlan et al 1995). FSH interacts with membrane receptor of the target cells, elicits a cascade of biochemical events preceded by the activation of adenyl cyclase and accumulation of intracellular c-AMP. Yet, phosphoinositide catabolism and calcium mobilization also may be involved (Richards and Hedin, 1988, Grasso and Richards 1990, Nikuka et al 1991). Stimulation of target cells by FSH is tightly regulated by a number of interacting mechanisms that ensure homeostasis for proper gonadal function and fertility.

The FSH receptor and its counterparts (LH and TSH receptors) are predicted to share a common motif composed of a large N-terminal extracellular motif linked to a hepta helical domain of seven membrane-spanning segments with extracellular intervening and intracellular loops. The extracellular domain is involved in hormone binding and is encoded by N-terminal multiple exons. The last exon encodes both the transmembrane and the intracellular domain (Heckert et al 1992). The extracellular domain has all the information necessary for high affinity hormone binding, the transmembrane domain helps in integrating the receptor into plasma membrane to form a functional unit (Sprengel et al 1990, Braun et al 1991) and the intracellular c-terminal tail participates in signaling. The cytoplasmic domain of the LH receptors has been implicated in phosphorylation-dependent desensitization mechanism, internalization and in proper membrane integration (Rodriguez et al 1992, Sanchez-Yagne et al 1992).

After the gonadotropin receptor c-DNAs were cloned from several species (Gromoll et al 1993, Kelton et al 1992, Minegish et al 1991, sprengel et al 1990, Yarney et al 1993), it became apparent that alternating spliced transcripts are co-expressed with the full-length receptor mRNA transcript (Themmen et al 1994, Misrahi et al 1996, O'Shaughnessy et al 1996). It is now becoming increasingly acceptable that other forms of receptors might exist. The discovery of alternative mRNA splicing transcripts for FSH (Gromoll et al 1992, Kelton et al 1992, Khan et al 1993, Khan et al 1997, Kraaij et al 1998), LH (Loosefelt et al 1989, Aatsinki et al 1992) and TSH (Libert et al 1990, Graves et al 1992) receptors in many species has led to the view that variants of receptors may be formed through tissue- or

cell-specific splicing of the parental pre-mRNA transcript. Functions of these putative receptor variants remain unclarified, but they are predicted to participate differentially in hormone binding and signaling mechanisms during different phases of reproduction. The transcripts coding for membrane-anchoring forms of FSH and LH receptor also have been described. They are apparently altered in specific exons of the extracellular domain of the receptor (Aatsinki et al 1992, Gromoll et al 1992). In the view of a recent report that an activated glycoprotein hormone TSH receptor coupling to as many as 10 different G proteins in thyroid membranes (Laugwitz et al 1996), the discovery of a transcript encoding a receptor with a variant cytoplasmic domain for the gonadotropin receptors might reveal an additional and important mechanism for regulation of hormone signal transduction (Yarney et al 1997).

On the other hand, tissue transglutaminase (tTG) is a novel, dual function protein having both transamidating activity and a role as a signal-transducing GTP-binding protein (Greenburg et al 1991, Nakaoka et al 1994). As a member of the transglutaminase family, tTG catalyzes a calcium-dependent acyl transfer reaction between the γ-carboxamide and peptide-bound glutamine residue and the ε-amino group of a peptide-bound lysine, or the primary amino group of a polyamine, yielding either an isopeptide bond or a (γ-glutamyl)polyamine bond, respectively (Greenburg et al 1991, Nakaoka et al 1994). This transamidating activity of tTG is inhibited by GTP, an effect that is reversed by an intrinsic GTPase activity of tTG (Lee et al 1989, Achyuthan et al 1987). GTP-bound tTG was subsequently shown to function as a signal-transducing GTP-binding protein (Gαh), which couples activated receptors to activate phospholipase C-δ1 (PLC-δ1), resulting in stimulation of this effector enzyme (Nakaoka et al 1994, Feng et al 1996). Thus, this intriguing protein can serve the cell in two apparently unrelated capacities, its role apparently determined by incompletely characterized intracellular regulators.

tTG is found in many different mammalian cells and tissues, and has been implicated as a participant in a vast array of physiological and pathological processes. In its capacity as a transamidating enzyme, tTG has been proposed to play an important role in bone development (aeschliman et al 1993), axonal growth and regeneration (Eitan and Schwartz 1993, Eitan et al 1994), modulation of cell adhesion (Gentile et al 1992, Borge et al 1996), differentiation and apoptosis (Hand et al 1993, Amendola et al 1996), and tumor growth and metastasis (Johnson et al 1994, Hettasch et al 1996). Recent studies have begun to elucidate the specific

roles of tTG in these different biochemical processes. For example, tTG is likely to be involved in the activation of both midkine, a heparin-binding growth/ differentiation factor, and interleukin 2 by catalyzing the formation of stable dimers (Eitan and Schwartz 1993, Eitan et al 1994, Kojima 1997). It has also been suggested that tTG contributes to the transforming growth factor-β activation process by cross-linking the large latent transforming growth factor-β complex to the extracellular matrix (Nunes et al 1997). Additionally, there are data to indicate that tTG is involved in stabilizing tissue during wound healing by cross-linking anchoring fibrils and, more specifically, collagen VII (Raghnath et al 1996).

In addition to catalyzing the formation of isodipeptide bonds, tTG in its role as a transamidating enzyme covalently incorporates polyamines into substrate proteins. Protein-polyamine conjugates have been detected in several tissue and cell lines (Piacentini et al 1988, Beninati and Folk 1988), and in vitro tTG incorporates polyamines into numerous proteins (Hohenadl et al 1995, Millerand Johnson 1995, Ballester et al 1996). Although functional changes resulting from tTG-catalyzed incorporation of polyamines into proteins have not been well defined, previous studies have shown that the covalent incorporation of polyamines into phospholipase A2 in vitro increases the enzyme activity (Cordella-Miele et al 1993).

In its capacity as a signal transducing GTP-binding protein, tTG has been designated as Gαh, a protein that forms noncovalent heterodimers with a 50-KDa protein (Nakaoka et al 1994, Baek et al 1996). Gαh has been shown to couple to α1-adrenoreceptor and modulate calcium influx through activating PLC-δ1 (Kang SK et al 2002). In cardiomyopathic heart tissue, the intrinsic activity of Gαh is decreased (Hwang et al 1996). The reason for the decrease in Gαh activity and GTP binding in the failing heart is unknown; however, it has been suggested that other proteins such as the 50-KDa protein that binds Gαh in a GTP-dependent manner, may be involved (Baek et al 1996). Interleukin 6 has been shown to induce tTG expression in hepatocytes (Suto et al 1993), and cAMP also induces tTG expression in cerebellar granule cells (Perry 1995). In many cells, retinoids are effective inducers of tTG expression (Davies et al 1985, Piacentini et al 1992, Benedetti et al 1996, Kosa et al 1995). In addition, the in vitro regulation of tTG activity by Ca++ and GTP has also been well documented (Achycethan and Greenberg 1987, Folk 1972, Folk and Finlayson 1977). Because tTG is apparently involved in multiple cellular processes, its expression and activation are likely to be

tightly regulated processes.

In the regard of FSH-Receptor interaction, calcium was reported to be required for maximal binding of FSH to receptor (Anderson and Reichert 1982). The binding of FSH to receptor was irreversible above 30℃ (Anderson et al 1983). A synthetic peptide amide corresponding to a region of the β-subunit of hFSH (hFSH-β-1~15) was shown to bind calcium (Santacoloma et al 1992) and induced calcium uptake by FSH receptor-containing liposomes (Grasso et al 1991). Furthermore, tTG was suggested to play a role in activation of Sertoli cells by FSH. This was thought to occur through modulation of activities of membrane and cytosolic components by tTG (Dias 1985). On the other hand, polyamines and tTG substrates interfered the fate of sequestered FSH in Sertoli cells but not the rate at which sequestration occurs (Diase 1986).

The administration of tTG inhibitors, bacitracin and N-ethylmaleimide, did not affect the FSH-receptor binding but enhanced the dissociation of [125I]-hFSH from its receptor. Reduced tTG activity paralleled increases in hormone-receptor dissociation (Grasso et al 1987). It was thus, speculated that protein cross-linking by tTG may be a mechanism to stabilize FSH-receptor complexes. In addition, purification of a light membrane fraction produced a FSH receptor-enriched fraction containing tTG activity, which cosolubilized with the FSH receptor and could be incorporated into liposomes (Grasso and Reichert Jr 1992). Calcium increased specific binding of FSH to receptor in a concentration-related manner, and the binding showed an increase in affinity (13.2 fold at 20 mM) of the receptor for FSH without significant change in receptor concentration. The authors concluded that calcium might stabilize FSH-receptor binding via activation of tTG-catalyzed isopeptide bond formation.

However, no single datum yet has been demonstrated to show the existence of covalently cross-linked FSH-receptor complexes (on SDS gel). It may be argued that the tTG activity in the FSH receptor enriched light membrane fraction assayed in the presence of calcium was not really “tTGs”. In the presence of high concentration of endogenous GTP, the tTG would act like Gαh. However, when GTP is depleted and Ca++ concentration rises, the Gαh would become a tTG. Therefore, those measured “tTG” activity may result from GTP-binding protein Gαh, an alternative coupling protein to FSH receptor/ receptor variant, assayed in vitro in the absence of GTP but the presence of calcium. Consequently, in the

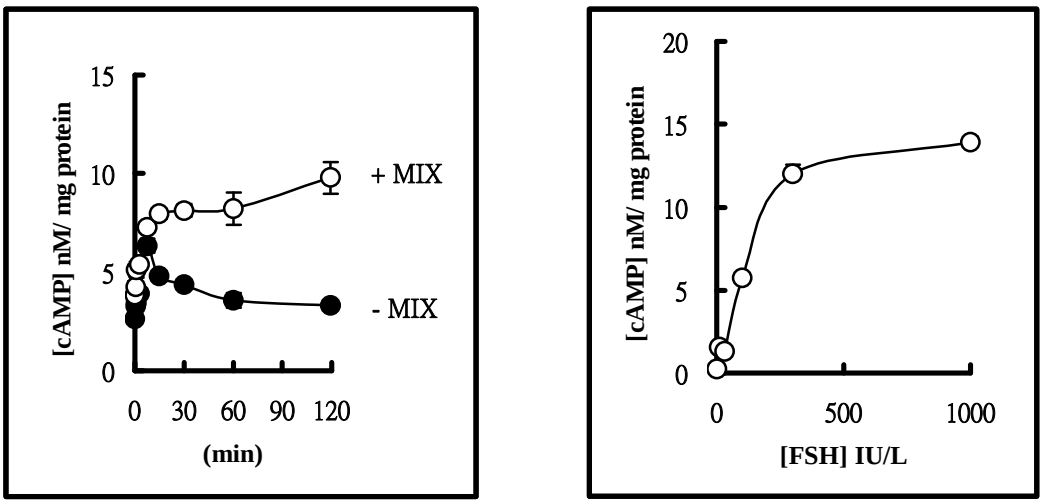
present study, efforts will be made either to demonstrate the existence of cross-linked FSH-receptor complexes or provide evidence for the involvement of Gαh in a portion of FSH actions.

四、 研究結果(2000-2002)

1. FSH-stimulated elevation of intracellular cAMP concentration in Sertoli cells in time- and dose-dependent manners.

FSH (300 IU/ L) was administered to 6 day-cultured Sertoli cells, which were pretreated with or without 1mM of Isobutyl-Methyl-Xanthine (MIX), a phosphodiesterase inhibitor, for 30 min at 34℃. After incubation, Sertoli cells were scrapped from culture plates in the extraction buffer and centrifuged at 20,000 x g at 4 ℃ for 30 minutes. The lysates were used to determine the intracellular cAMP concentration by the commercial cAMP EIA kit. The data showed that the FSH-stimulated elevation and the MIX-dependent accumulation of intracellular cAMP concentration were appeared after 10-min treatment of FSH (Fig. 1a). The FSH-stimulated dose-dependent accumulation of intracellular cAMP concentration reached the maximal levels when Sertoli cells were treated with 300-1000 IU/ L of FSH (Fig. 1b). The data from three independent experiments were calculated with SigmaPlot software and the values presented in the graph are means ± SD.

Figure 1a. **1b.**

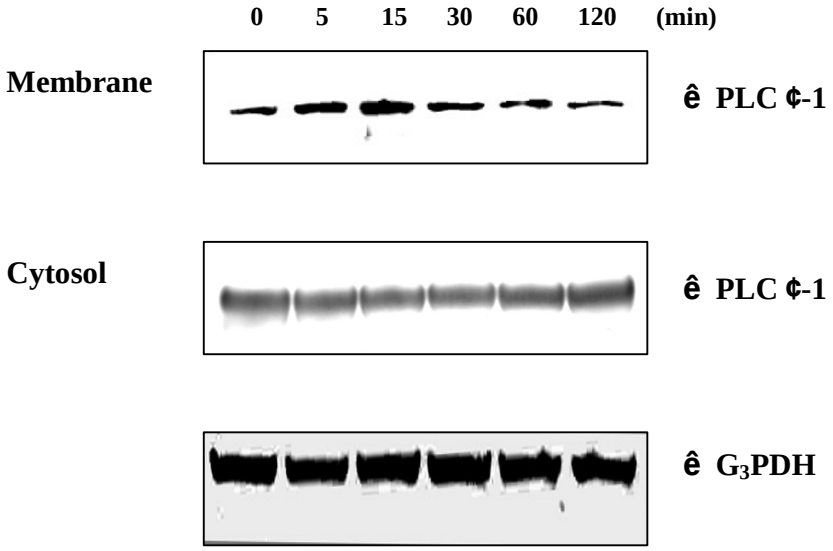


2. FSH-induced PLC ¢-1 translocation from cytosol to membrane in Sertoli cells

Sertoli cells were cultured for 6 days and then treated with 300 IU/ L of FSH at the indicated time intervals. After incubation, Sertoli cells were scrapped from culture plates in extraction buffer and the cell lysates were centrifuged at 300 xg at 4℃ for 10

minutes. The supernatants were transferred to new eppendorf tubes and then centrifuged at 50,000 xg at 4 ℃ for 60 minutes. The pellets (light membrane) and supernatant (cytosol) were collected and then analyzed by western blotting using PLC δ-1 specific antibody. The data showed that FSH-stimulated PLC δ-1 translocated from Sertoli cell cytosol to cell membrane after 30-min treatment (Fig. 2). In this case, G3PDH was used as an internal control of the loaded protein concentration.

Figure 2.

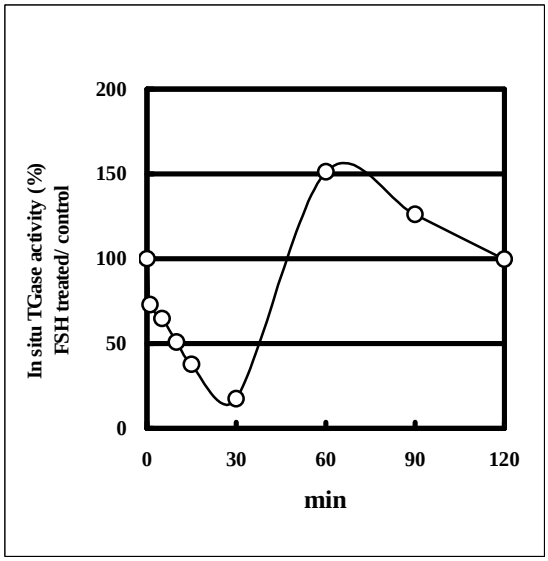


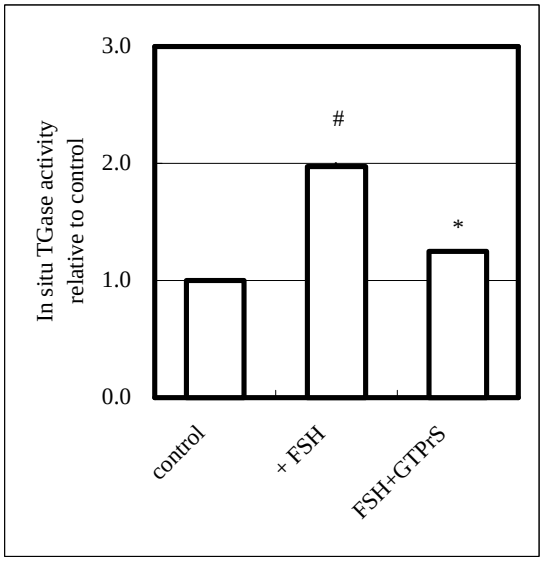
3. <u>FSH-induced biphasic change in tTGase activity of Sertoli cells by in situ tTGase activity analysis</u>

FSH (300 IU/ L) was administered to 6 day-cultured Sertoli cells, which were pretreated with 1mM of 5-biotinamido-pentylamine, a tTG substrate, for 30 min at 34℃. After incubation, Sertoli cells were scrapped from culture plates in the extraction buffer and centrifuged at 20,000 x g at 4℃ for 30 minutes. The supernatants were used to measure the biotinylated proteins, which referred as in situ intracellular tTG activity, by using HRP-conjugated streptavidin system. The data showed that tTG activity decreased after 30-min FSH administration and reversed to original activity after 60-min of FSH treatment in Sertoli cells (Fig. 3a). The pretreatment of Sertoli cells with GTPγS abolished the FSH-stimulated tTG activity by 1h of FSH treatment (Fig 3b). These results reveal that FSH can trigger tTGase protein to undertake the G protein function in Sertoli cells. The data from three independent experiments were calculated with SigmaPlot software. (* compared to # , $p < 0.001$; # compared to control, $p < 0.001$)

Figure 3a. **3b.**

In situ TGase activities in cytosol fraction after FSH treatment

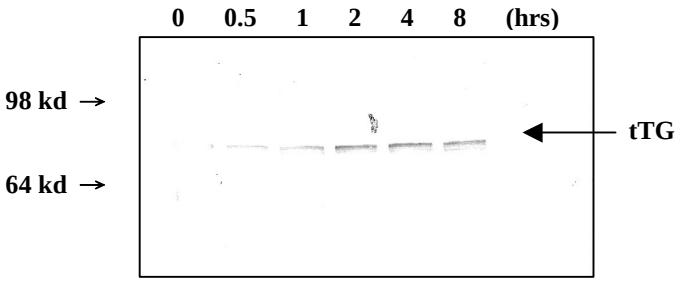




4. GTP-agarose-absorbed G proteins in membrane fractions of Sertoli cells recognized by anti-tTGase antibodies

The 6-day cultured Sertoli cells were treated with 300 IU/ L of FSH at the indicated time intervals. After incubation, Sertoli cells were scrapped from culture plates in extraction buffer and the cell lysates were centrifuged at 300 xg at 4℃ for 10 minutes. The supernatants were transferred to new eppendorf tubes and then centrifuged at 50,000 xg at 4 ℃ for 60 minutes. The pellets (light membrane) were incubated with GTP-agarose and then resolved by western blotting analysis using tTGase specific antibody. The data show that the GTP-agarose-absorbed proteins were recognized by monospecific anti-tTGase antibody (which interacts only with tTGase, NEOMARKERS, INC.) (Fig. 4) and revealed that a maximal concentration in the Sertoli cell membrane fraction after 4-hour of treatment with FSH.

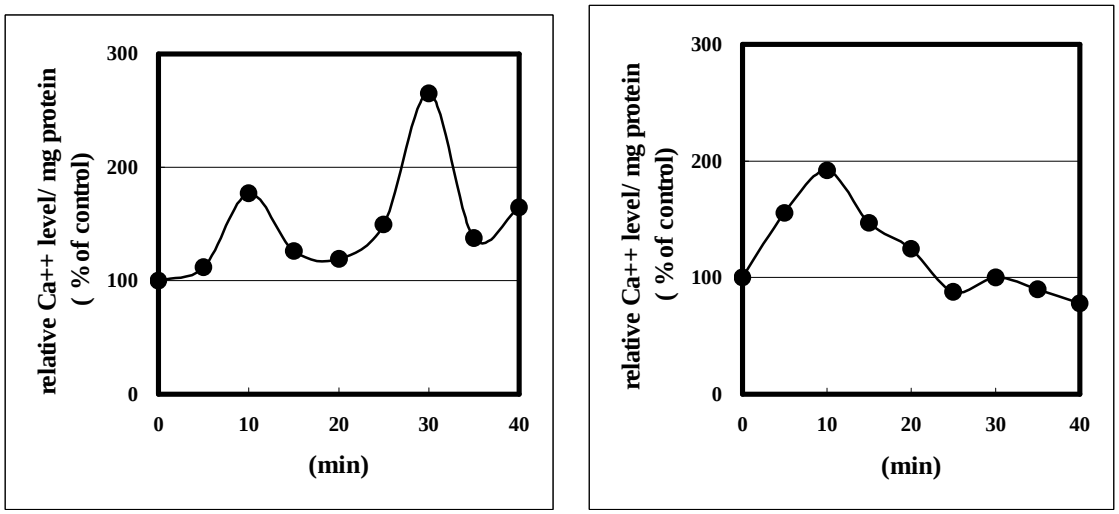
Figure 4.



5. FSH-stimulated intracellular calcium oscillation in Sertoli cells

After 6 days of culture, Sertoli cells were cultured in the DMEM/ F-12 media with or without calcium and then incubated with 1 uM Fura-2-AM for 30 min at 34℃. Subsequently, Sertoli cells were treated with 300 IU/ L of FSH at the indicated time intervals. FSH treated-Sertoli cells were scrapped from culture dishes in the extraction buffer and the lysates were centrifuged at 300 xg for 15 minutes at 4℃. Next, the supernatants were transferred to new eppendorf tubes and then centrifuged at 10,000 xg at 4 ℃ for 10 minutes. The supernatants (cytosol) were analyzed by Hitachi F-4500 fluorospectromemter (Ex=340 nm/ Em=500 nm). The data from three independent experiments were calculated with SigmaPlot software. The data show that there is a biphasic profile of FSH-induced intracellular calcium elevation when Sertoli cells were cultured in regular DMEM/ F12 medium containing 2.9 mM Ca^{++}(Fig. 5a). However, FSH-induced second phase of intracellular calcium elevation was disappeared in calcium-free DMEM/ F-12 medium (Fig. 5b). Those indicated that FSH-induced intracellular calcium releasing appears in the first phase, while FSH-induced extracellular calcium influx represents in the second phase.

Figure 5a. **5b.**

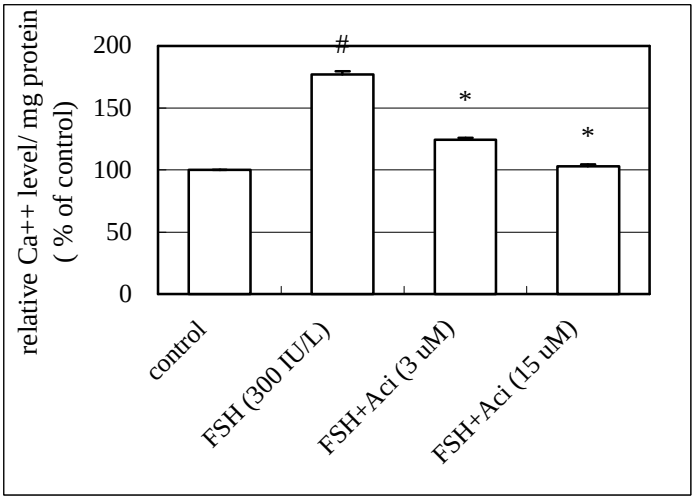


6. Adenyl cyclase inhibitor decreases FSH-induced intracellular calcium release in rat Sertoli cells

After 6 days of culture, Sertoli cells were incubated and pretreated with 1 uM Fura-2-AM and adenyl cyclase inhibitor (3 uM and 15 uM), 2', 5'-deoxyadenosine, for 30 min at 34℃. Subsequently, Sertoli cells were treated with 300 IU/ L of FSH for 10 min. FSH treated-Sertoli cells were scrapped from culture dishes in the extraction buffer and the lysates were centrifuged at 300 xg for 15 minutes at 4℃. Next, the supernatants were transferred to new eppendorf tubes and then centrifuged at 10,000 xg at 4 ℃ for 10 minutes. The supernatants (cytosol) were analyzed by Hitachi F-4500 fluorospectromemter (Ex=340 nm/ Em=500 nm). The data from three independent experiments were calculated with SigmaPlot software. The data show that 2', 5'-deoxyadenosine dose-dependently suppressed FSH-induced

intracellular calcium release (Fig. 6). (* compared to # , p< 0.01; # compared to control, p< 0.01)

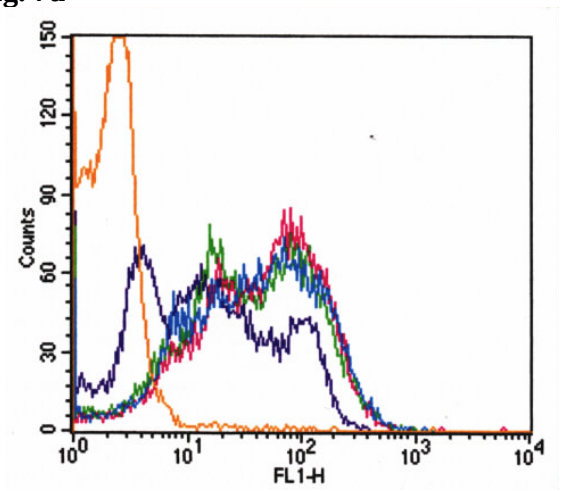
Fig. 6



7. FSH elevates N-cadherin expression in time dependent manner in rat Sertoli cells

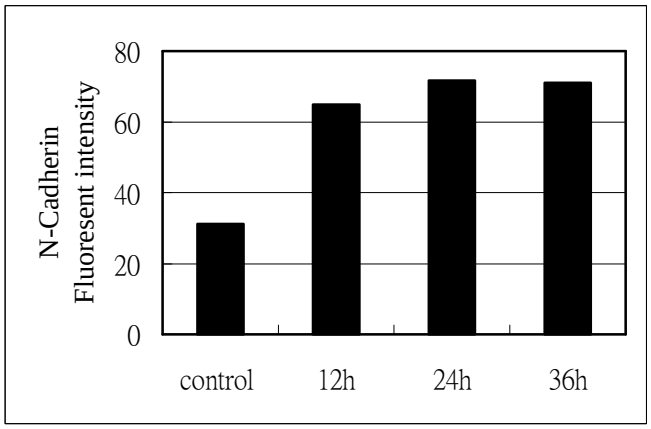
After 6 days of culture, Sertoli cells were treated with 300 IU/ L of FSH at the indicated time. Subsequently, Sertoli cells were collected in the phosphate-buffer saline (PBS). Next, Sertoli cells ($2x10^5$) were stained with N-cadherin specific Ab and then analyzed by flow cytometry. The N-cadherin expression was represented by fluorescent intensity (FI) as shown in Fig. 7a. The Geometric mean of FI was calculated and the relative folds of fluorescent intensity were shown by vertical bar chart in Fig. 7b. The data show that FSH enhances N-cadherin expression by 2-fold elevation in rat Sertoli cells.

Fig. 7a



Control	Purple
12h	Green
24h	Red
36h	Blue
Ab isotype control	Golden

Fig 7b



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六、未來研究方向

Further efforts will be made to define the importance of Gh-PLC δ-1 pathway in the physiologic functions of Sertoli cells in response to FSH-treatment. In addition, efforts will also be made to resolve how many different G protein subfamilies may be involved in the FSH-triggered signal transduction pathway in Sertoli cells.

國科會多年期計畫期中報告

組織性轉麩胺醯在性腺激素作用中所扮演的角色（2/2）

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行政院國家科學委員會補助專題研究計畫成果報告

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