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一、英文摘要

Follicle-stimulating hormone (FSH) is known to activate Gs/cAMP signaling pathway in Sertoli cells to support spermatogenesis. However, the molecular mechanism of FSH-induced Gs/cAMP-independent Ca^{2+}-influx in Sertoli cells is not clear.

In this study, FSH was found to efficiently induce the activations of Gαh and PLC-δ1 proteins in rat Sertoli cells. Subsequent study showed that the T-type Ca^{2+}-channel mediated FSH-evoked immediate Ca^{2+}-influx of Sertoli cells as verified by using its specific inhibitor, Mibefradil. To verify the involvement of Gαh/PLC-δ1 pathway in the FSH-induced Ca^{2+}-influx of rat Sertoli cells, a peptide, TIPWNSLKQ GYRHVHLL, of PLC-δ1 was synthesized to compete with endogenous PLC-δ1 in its interaction with Gαh. The data showed that the synthetic peptide of PLC-δ1 dose-dependently inhibited the FSH-induced Ca^{2+} influx and PLC-δ1 activation in rat Sertoli cells. However, the pretreatment of rat Sertoli cells with NF-449 was shown to enhance the FSH-induced PLC-δ1 activation. On the other hand, the adenyl cyclase inhibitor, 2',5'-dideoxyadenosine, but not the synthetic peptide of PLC-δ1, reduced the FSH-induced intracellular cAMP accumulation in rat Sertoli cells. Furthermore, neither inactivated nor activated forms of Gαh were able to interact with FSH receptor directly.

In conclusion, the FSH-induced immediate T-type Ca^{2+}-influx of Sertoli cells is modulated by an alternative Gαh/PLC-δ1 pathway and might be independent of the classical FSH receptor. The data not only clarify the mechanism of FSH-induced Ca^{2+}-influx, but also contribute to the discovery of a novel FSH signaling pathway via the activation of Gαh instead of Gs in rat Sertoli cells.

Keywords: tissue transglutaminase, Gαh, PLC-δ1, Ca^{2+}-influx, FSH, Sertoli cells

二、中文摘要

組織型轉麩胺酶(Tissue transglutaminase)在細細胞中是一種多功能蛋白，它能夠以G-蛋白(G-protein)的形式(稱為 Gαh)活化磷酸脂激酶 C-δ1 (phospholipase C-δ1, PLC-δ1)，它們的交互作用已經被證實與細胞外鈣引入(Ca^{2+}-influx)的分子機制有關。另一方面，促濾泡成長激素(Follicle-stimulating hormone, FSH)在 Sertoli 細胞(Sertoli cells)中所誘發之外鈣引入，其分子機制至今仍未被釐清，因此，Gαh 與 PLC-δ1 的交互作用是否參與 FSH 在 Sertoli cells 中所誘發之外鈣引入的過程為這篇論文主要探討的方向。

實驗結果證實 FSH 作用於 Sertoli cells 後，會活化細胞內之 Gαh 與 PLC-δ1。為了證實 Gαh 與 PLC-δ1 的交互作用對於 FSH 在 Sertoli cells 中所誘發之外鈣引入的過程是必要地，因此，我們進一步地合成一段 PLC-δ1 之 720 至 736 氨基酸序列的蛋白片段，作為阻斷 Gαh 與 PLC-δ1 交互作用的競爭型抑制劑。實驗結果顯示這段 PLC-δ1 的蛋白片段會明顯的抑制 FSH 在 Sertoli cells 中所誘發之外鈣引入，但是對於 FSH 所引發之細胞內 cAMP 的增加，卻不造成影響。另一方面，實驗中利用 T-型鈣離子通道(T-type Ca^{2+} channel)之抑制劑- Mibefradil，發現 FSH 在 Sertoli cells 中所誘發外鈣引入是經由細胞膜上之 T-型鈣離子通道。然而，利用專一性抑制劑阻斷已知與 FSH 接受器(receptor)結合之 Gs 蛋白的活化，發現並不會影響 FSH 誘發之 Gαh 與 PLC-δ1 的活化及外鈣的引入。且最後的實驗結果指出無論是活化型或不活化型之 Gαh，都不會與一般所知之 FSH 接受器作用。

綜合以上結果，我們發現 Gαh 與 PLC-δ1 的交互作用參與了 FSH 在 Sertoli cells 中所誘發外鈣引入的分子機制，而且這一條 FSH 在 Sertoli cells 中所引發之新穎的訊息傳遞路徑是與一般已知之 Gs/cAMP 之路徑無關。此一研究結果，對於探討 FSH 在 Sertoli cell 中未知之作用，新闢了一條研究的方向。另外，Gαh 不會與傳統之 FSH 接受器作用的結果，也提供了進一步研究 Sertoli cell 中嶄新 Gαh 結合型之 FSH 接受器的可行性。

關鍵詞：組織型轉麩胺酶、Gαh、磷酸脂激酶 C-δ1、外鈣引入、促濾泡成長激素、Sertoli 細胞

三、研究背景及目的

Follicle-stimulating hormone (FSH) is released from pituitary gland and primarily regulates follicular development in the ovary and spermatogenesis in the testis (McLachlan et al., 1995). Upon interacting with its receptor on the target cells, FSH elicits a cascade of biochemical events preceded by the activation of Gs/adenyl cyclase pathway and the accumulation of intracellular cAMP (Gorczynska et al., 1994). Stimulation of target cells by FSH is tightly regulated by a number of interacting mechanisms that ensure homeostasis for proper gonadal function and fertility. On the other hand, several previous studies demonstrated that the interaction of FSH and its receptor induced a rapid Ca^{2+}-influx (Touyz et al., 2000; Grasso et al., 1992; Grasso and Reichert, Jr., 1989), which might be associated with phosphatidyl- inositol-dependent PLC (PI-PLC)-dependent pathway (Filippini et al., 1994), but not Gs/adenyl cyclase-dependent pathway (Grasso and Reichert, Jr., 1989) in Sertoli cells. Despite near two decades of studies, no working mechanism has been established for the FSH-induced Ca^{2+}-influx in Sertoli cells.

Ca^{2+}-influx has been known to be required for protein secretion from excitable cell types (Bosco et al., 2001; Gardella et al., 2000; Kallenberg, 2000; Satin, 2000). Recently, it was reported that FSH-induced Ca^{2+}-influx was required for the exocytosis of secretory proteins from Sertoli cells (Taranta et al., 1997). Particularly, the secretion of inhibins from Sertoli cells is thought to be important for the negative regulation of FSH-release from pituitary gland (Berensztein et al., 2000; Taranta et al., 1997). Previous studies on the distribution of calcium channels in Sertoli cells using various agonists and blockers revealed that all T (transient)-, L (long-term)-, and N (neural)-type Ca^{2+} channels might simultaneously exist in Sertoli cells (Taranta et al., 1997; Lalevee et al., 1997; D'Agostino et al., 1992;Gorczynska and Handelsman, 1991). However, which kind of Ca^{2+} channels modulating the FSH-induced immediate Ca^{2+}-influx in Sertoli cells remains unclear.

Tissue transglutamiase (tTG) has been known to possess multiple functions in various cell types. In addition to its transamidating activity, tTG also acts as ATPase, GTPase and GTP-binding protein, designated as Gαh. Recently, a 50 kD protein, Gβh, was shown to modulate GTP-binding activity of Gαh and was identified to be calreticulin later (Lee et al., 2003). Although tTG was primarily identified by its transamidating activity, the recent study demonstrated that tTG mainly acts as Gαh in the physiological condition (Mhaouty-Kodja, 2004;Zhang et al., 1998). The increased tTG activity has been demonstrated to be related to the pathological progression (Mhaouty-Kodja, 2004). Phospholipase C-δ1 (PLC-δ1) has been identified to be a down-stream effector of Gαh in the intracellular signal transduction pathway (Feng et al., 1996; Das et al., 1993; Baek et al., 1993). The interaction of Gαh and

PLC-δ1 has been found to modulate the α_{1B}-adrenoreceptor- induced Ca^{2+}-influx in a smooth muscle cell line, DDT1-MF2 cells (Kang et al., 2002).

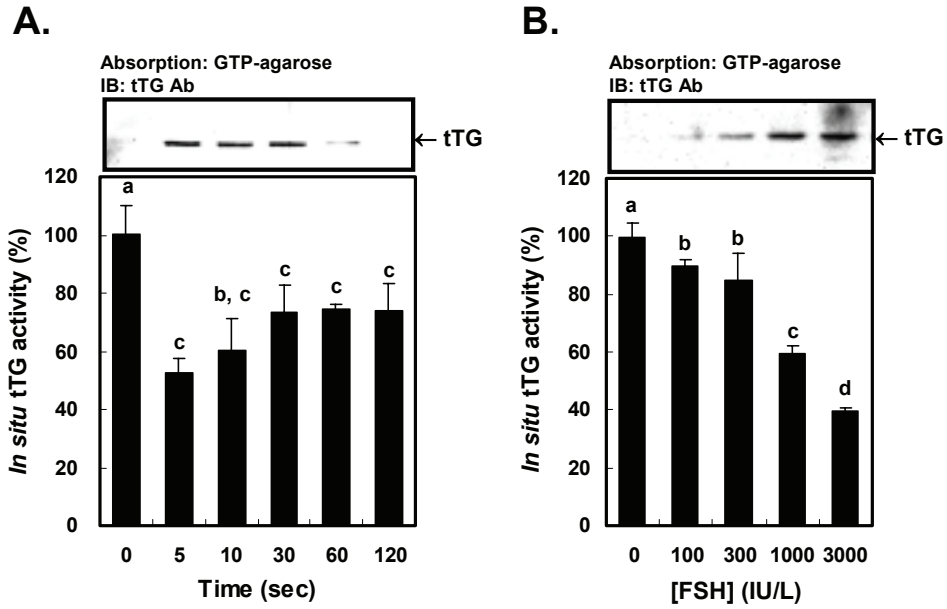
Dias (1985) suggested that ttTG plays a role in activating Sertoli cells by FSH through modulating the activities of membrane and cytosolic components. Polyamines and tTG substrates interfered the fate of sequestered FSH in Sertoli cells but not the rate at which sequestration occurs (Dias, 1986). The administration of tTG inhibitors, bacitracin and N-ethylmaleimide, did not affect the FSH-receptor binding but enhanced the dissociation of [^{125}I]-hFSH from its receptor. Reduced tTG activity was found to parallel the increase in hormone-receptor dissociation (Grasso et al., 1987). These authors speculated that protein cross-linking caused by tTG might be required for the stabilization of FSH-receptor complexes. Alternatively, we proposed that tTG might act as a novel FSH receptor-coupled G protein.

In this study, it was intended to establish whether Gαh might be a distinct class of G-protein other than Gs in FSH action. It was also attempted to clarify the molecular mechanism of the FSH-induced Ca^{2+}-influx in rat Sertoli cells and identify the type of Ca^{2+}-channel activated by FSH. The results unambiguously demonstrated that Gαh/PLC-δ1 pathway is involved in the Gs-independent, FSH-induced immediate, T-type Ca^{2+}-influx in rat Sertoli cells.

四、研究結果

1. <u>The time- and dose-dependent effects of FSH on GTP-bound Gαh activity and *in situ* tTG activity of rat Sertoli cells</u>. Rat Sertoli cells were incubated with FSH (1000 IU/L) for the indicated time intervals, or treated with various doses of FSH for 10 seconds. The GTP-absorbed Gαh protein in the cell membrane fraction was analyzed by SDS-PAGE followed by Western blot analysis with a tTG specific antibody (A and B, tops). On the other hand, the *in situ* tTG activity was determined by incubating Sertoli cells with 1 mM 5'-biotinamido-pentylamine for 40 min at 34°C prior to the treatment with FSH for various time intervals (A, bottom) or with various doses of FSH (B, bottom). The tTG activity was assessed as described in the "Methods". The data from triplicate experiments were analyzed by One Way ANOVA and Duncan Multiple Range test. Data shown in Figure 1A and 1B (bottoms) represent means ± SE (n = 4). Different alphabets in the adjacent order represent the significance of statistical differences between the adjacent values at $p< 0.05$.

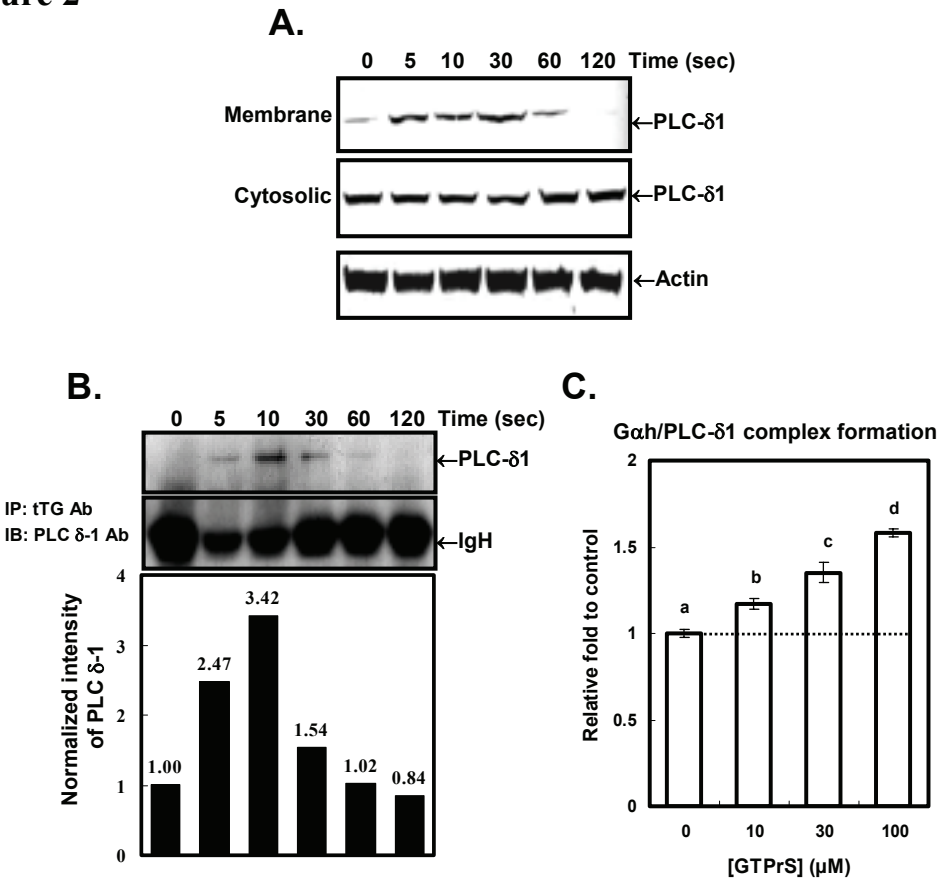
Figure 1



2. <u>The effects of FSH on PLC-δ1 translocation and the formation of Gαh/ PLC-δ1 complex</u>. After rat Sertoli cells incubated with FSH (1000 IU/L) for various time intervals, the extracted cell membrane and cytosolic fractions were employed to determine the PLC-δ1 translocation (A). Actin was used as the internal control for relative cytosolic protein loading. In addition, cell membrane fraction was also immunoprecipitated with Gαh antibody to analyze the formation of Gαh/PLC-δ1 complex by Western Blot analysis with PLC-δ1 antibody. Figure 2B (bottom) shows the normalized, relative to immunoglobulin heavy chain

(IgH), intensities of PLC-δ1 values which were peaked at 10 sec after FSH-treatment. Furthermore, the whole cell lysates of Sertoli cells were preincubated with various concentrations of GTPγS and the formation of Gαh/PLC-δ1 complexes were also immunoprecipitated with Gαh antibody followed by ELISA assay with a PLC-δ1 specific antibody. Data shown in Figure 2C represent means ± SE (n = 3). Different alphabets in series indicate the significance of statistical differences between the adjacent values at $p < 0.05$.

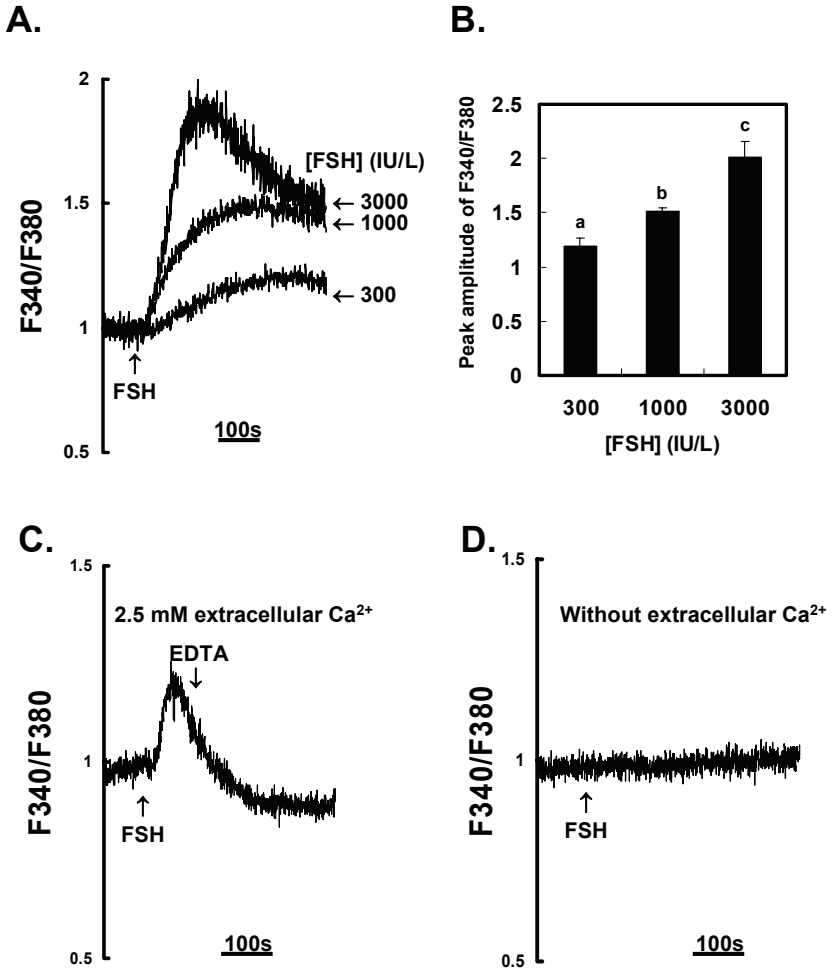
Figure 2



3. <u>The characterization of FSH-induced cellular Ca^{2+} elevation in rat Sertoli cells</u>. Rat Sertoli cells were preincubated with Fura-2-AM (5 μM) for 40 min at 34 °C and then subjected to intracellular Ca^{2+} determination. FSH was administrated at 100 sec since the commence of intracellular Ca^{2+} determination. The intracellular Ca^{2+} levels of rat Sertoli cells were dose-dependently increased after FSH treatment (A and B). Subsequently, the FSH (3000 IU/L)-induced intracellular Ca^{2+} increase was abolished by injecting 2.5 mM of EDTA at 150 sec (C). No FSH (3000 IU/L)-induced intracellular Ca^{2+}-increase was observed when Sertoli cells were incubated in Ca^{2+}-free media (D). The triplicate data as shown in A were analyzed by One-way ANOVA and Duncan Multiple Range test. The column heights represent means ± SE (n = 3) of peak amplitudes (B). Different alphabets in serial sequence

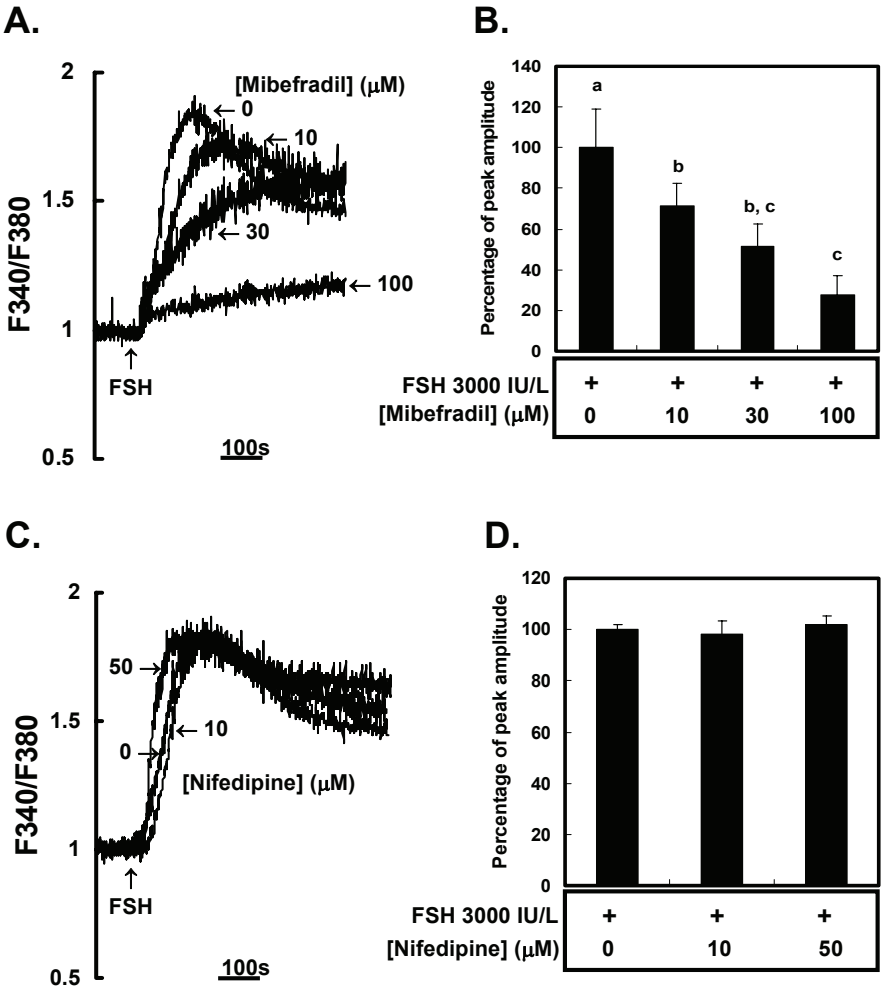
indicate the significance of statistical differences between the adjacent ones at $p < 0.05$.

Figure 3



4. <u>The identification of Ca^{2+} channel type for the FSH-induced Ca^{2+}-influx in rat Sertoli cells</u>. Rat Sertoli cells were preincubated with Fura-2-AM (5 μM) and Mibefradil (10, 30 and 100 μM), or Nifedipine (10 and 50 μM), a selective inhibitor of T-type Ca^{2+}-channel or an L-type Ca^{2+}-channel blocker, respectively, for 40 min at 34 °C prior to the intracellular Ca^{2+} determination. FSH (3000 IU/L) was administrated at 100 sec after the onset of the Ca^{2+} determination process. The FSH-evoked Ca^{2+}-influxes in rat Sertoli cells were dose-dependently inhibited by the pretreatment with Mibefradil (A and B), but not with Nifedipine (C and D). Figures 4 B and 4D summarized the triplicate data of 4A and 4C after analyzed by One-way ANOVA and Duncan multiple range test. The column heights represent means ± SE of peak amplitudes. Different alphabets in serial sequence indicate the significance of statistical differences between the adjacent ones at $p < 0.05$.

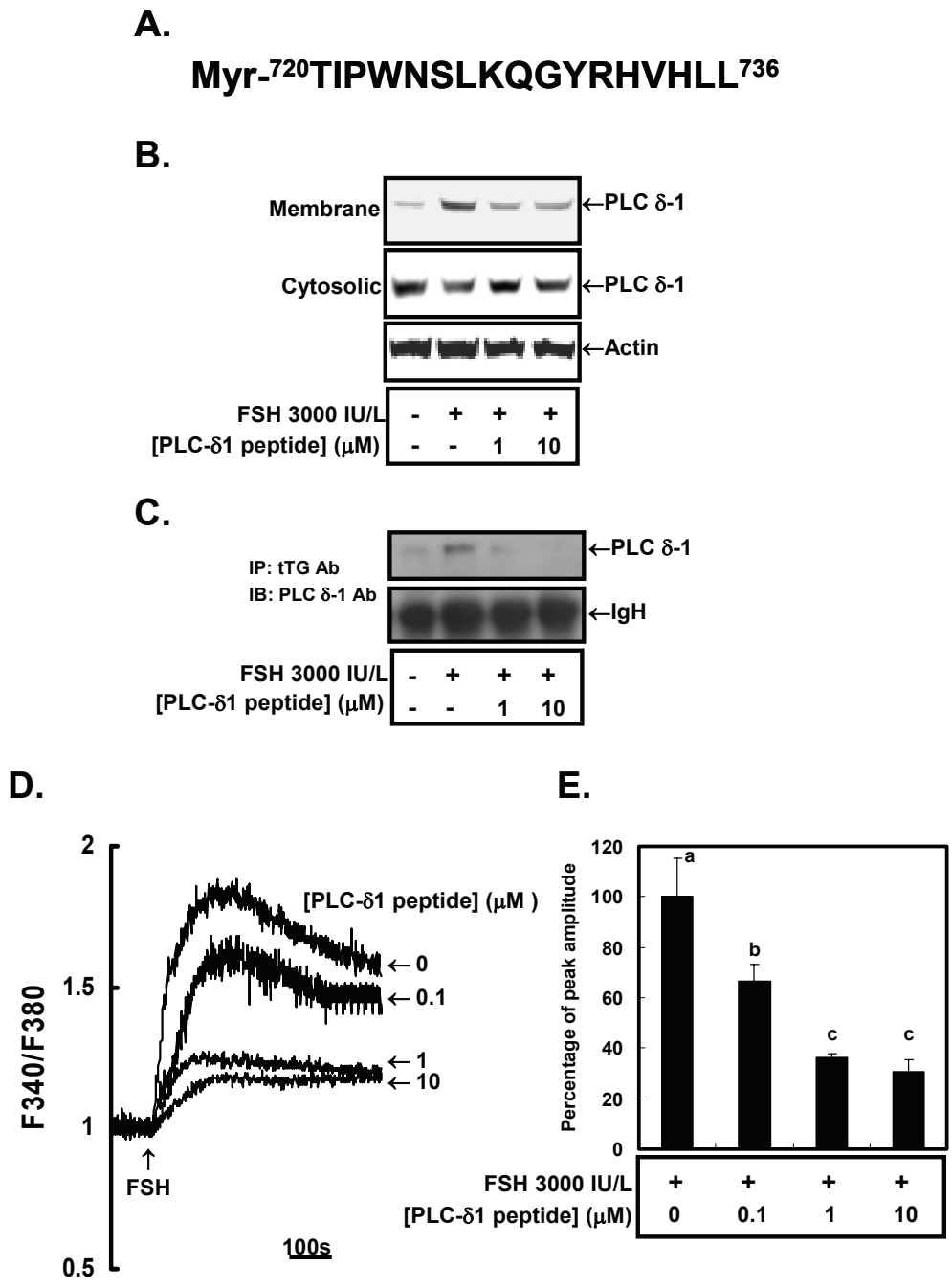
Figure 4



5. <u>The essential role of Gαh/PLC-δ1 complex formation in the FSH-induced Sertoli cell Ca^{2+} influx</u>. Prior to the administration of FSH (1000 IU/L), rat Sertoli cells were pretreated with the synthetic PLC-δ1 peptide (A) at designated concentrations for 30 min at 34°C. The FSH-induced PLC-δ1 translocation from cytosol to cell membrane was determined as described in "Methods" (B). Actin was used as the internal control for relative cytosolic protein loading. Membrane fractions of rat Sertoli cells were subsequently immuno-precipitated with Gαh antibody, followed by Western Blot analysis with a PLC-δ1 specific antibody to analyze for the formation of Gαh/PLC-δ1 complexes (C). IgH was used as an indicator for the relative protein levels of immunoprecipitated products. On the other hand, rat Sertoli cells were preincubated with Fura-2-AM (5 μM) and various concentrations of the synthetic PLC-δ1 peptide for 40 min at 34 °C prior to the determination of rate Sertoli cell intracellular Ca^{2+} in response to FSH treatment (D). The triplicate data of D were analyzed by One-way ANOVA and Duncan Multiple Range test. The column heights represent means ± SE (n = 3) of peak amplitudes (B). Different alphabets in serial sequence indicate the

significance of statistical differences between the adjacent ones at $p < 0.05$.

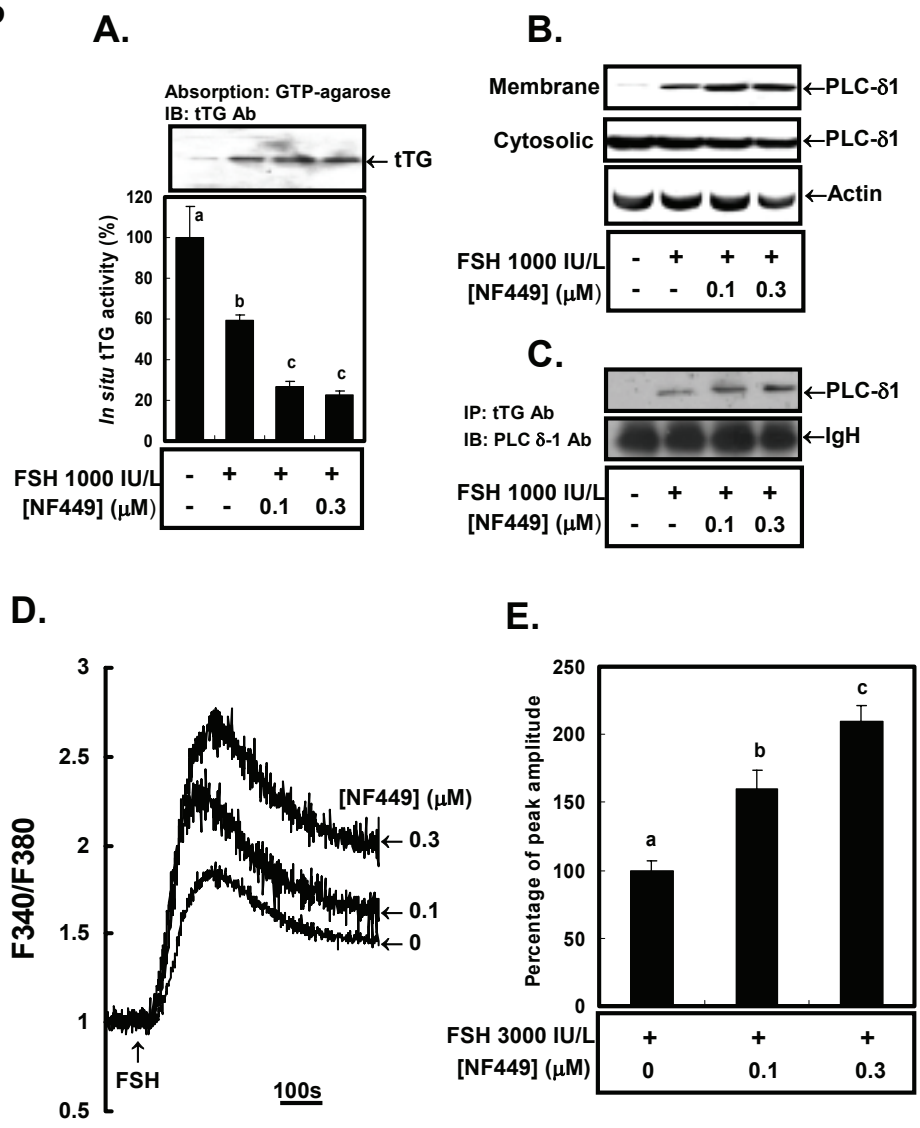
Figure 5



6. <u>The independency of Gαh/PLC-δ1 activation and Ca^{2+} influx to the calssical Gs-dependent signaling pathway in FSH-activated Sertoli cells</u>. Sertoli cells were preincubated with designated doses of NF-449, a Gs protein specific inhibitor, at 34°C for 40 min prior to the administration of FSH (3000 IU/L) for 10 sec. The membrane fractions of

Sertoli cells were used to analyze for the levels of GTP-absorbed Gαh protein (A, top). The whole cell lysates were employed to determine the *in situ* tTG activity (A, bottom). Subsequently, the PLC-δ1 levels in the cell membrane and cytosolic compartments were analyzed by Western Blotting with a PLC-δ1 specific antibody to determine the translocation of PLC-δ1 (B). Actin was used as the internal control for relative cytosolic protein loading. The cell membrane fraction of Sertoli cells was immunoprecipitated with Gαh antibody followed by Western Blot analysis with a PLC-δ1 specific antibody to analyze for the formation of Gαh/PLC-δ1 complexes (C). IgH was used as an indicator for the relative protein level of immunoprecipitated products. Furthermore, the data show that FSH-induced Ca^{2+} influxes of rat Sertoli cells were enhanced by NF-449 in a dose-dependent manner (D and E). Data shown in bottom panel of Figure 6A represent means ± SE (n = 4). The data in Figure 6E was means of triplicate results of Figures 6D. The column heights represent means ± SE (n = 3) of peak amplitudes. Different alphabets in series indicate the significance of

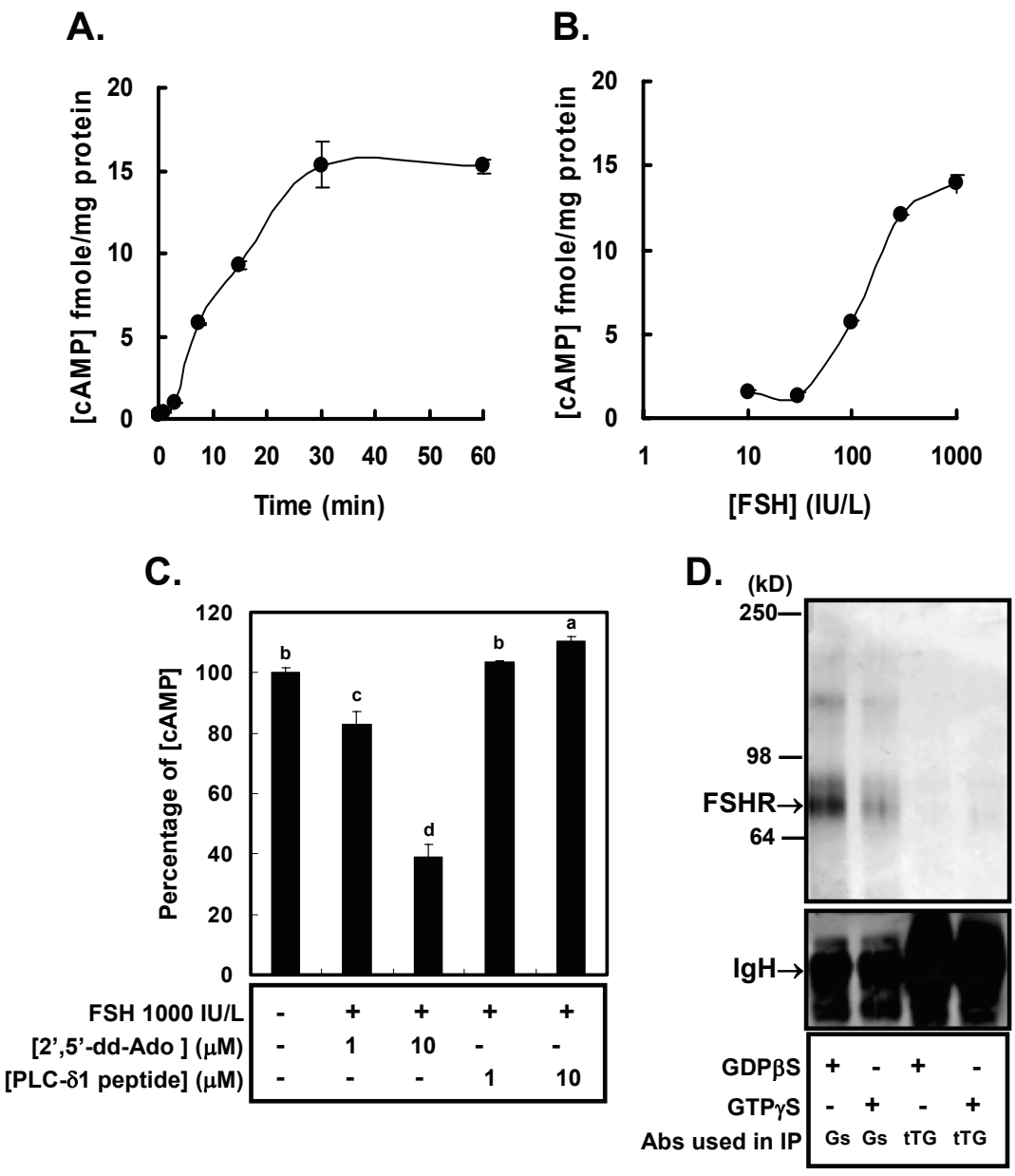
Figure 6



statistical differences between the adjacent ones at $p < 0.05$ after analyzed by One-way ANOVA and Duncan multiple range test.

7. <u>The effects of 2',5'-dideoxyadenosine and synthetic PLC-δ1 peptide on FSH-induced intracellular cAMP accumulation</u>. Rat Sertoli cells were preincubated with either 0.2 mM of phosphodiesterase inhibitor, isobutyl-methylxanthine, or adenyl cyclase inhibitor 2',5'-dideoxyadenosine (2',5'-dd-Ado), or the synthetic PLC-δ1 peptide for 30 min at 34°C prior to the treatment with FSH (1000 IU/L) for 30 min. the FSH (300 IU/L)-induced intracellular cAMP elevation reached the maximal level at 30-min of treatment (A). The effect of 30-minute FSH treatment on intracellular cAMP accumulation in rat Sertoli cells exhibited a linear response at the range of 30 to 300 IU/L (B). The FSH-induced intracellular cAMP

Figure 7



accumulation in rat Sertoli cells was dose-dependently inhibited by 2',5'-dideoAd. A slight but significant enhancement of cellular cAMP was induced by the synthetic PLC-δ1 peptide treatment at 10 μM (C). The column heights represent means ± SE (n = 3). Column with different alphabets in the adjacent series indicate the significance of differences between the adjacent ones at $p < 0.05$. In addition, 100 μg of the cytosolic and membrane extracts of rat Sertoli cells were pretreated with either 0.1 mM of GDPβS or GTPγS to irreversibly inactivate or activate all G proteins for 1 hour at 4°C. After immunoprecipitation with Gs or tTG specific antibodies, the precipitates were analyzed and blotted with FSH receptor antibody by SDS-PAGE/Western blot analysis. The result shows that classical FSH receptor was abundantly co-immuno- precipitated with Gs protein, particular in GDPβS-bound Gs protein. However, the classical FSH receptor interacted with neither GDPβS- nor GTPγS-bound tTG/Gαh proteins as revealed by the immunoprecipitation process. IgH represents the immunoglobulin heavy chain and was used as an indicator for the amount of protein loaded per well.

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