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Follicle-Stimulating Hormone-Induced Gαh/Phospholipase C-δ1 Signaling Mediating a Noncapacitative Ca^{2+} Influx through T-Type Ca^{2+} Channels in Rat Sertoli Cells

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Our previous study demonstrated that FSH-induced immediate Ca^{2+} influx in rat Sertoli cells (SCs) is mediated by the Gαh/phospholipase C-δ1 (PLC-δ1) signaling pathway. As to which Ca^{2+} channel is responsible for such Ca^{2+} influx was not understood. In this study, thapsigargin triggered an in-store calcium release and evoked a 1.5-fold elevation of intracellular Ca^{2+} in Ca^{2+}-free media, whereas FSH exhibited no effect. The readdition of $CaCl_2$ (2.5 mM) to FSH-pretreated or thapsigargin-sensitized SCs in Ca^{2+}-free media immediately elicited a rapid Ca^{2+} influx or a 2-fold increase of second intracellular Ca^{2+} elevation, respectively. The addition of Ca^{2+} chelator EGTA (0.2 mM) reduced the FSH-induced elevation of intracellular Ca^{2+} in SCs incubated with $CaCl_2$. However, pretreatment with dantrolene (25 μM), which inhibits in-store calcium release, did not affect the FSH-induced elevation of intracellular Ca^{2+}. $NiCl_2$ (10 μM), a T-type calcium channel blocker, abolished the FSH-induced SC Ca^{2+} influx. Furthermore, mibefradil (10 and 100 μM), another specific blocker for T-type Ca^{2+} channels, dose-dependently suppressed the FSH-induced Ca^{2+} influx. In contrast, nifedipine (10 and 50 μM) or ω-conotoxin GVIA (100 and 500 nM), blocker of L- or N-type Ca^{2+} channels, respectively, did not affect the FSH-induced SC Ca^{2+} influx. On the other hand, FSH-induced Ca^{2+} influx was significantly reduced by pretreatment of SCs with myristoylated synthetic peptide (0.1 and 1 μM) of PLC-δ1 fragment TIPWNSLKQGYRHVHLL but not affected by 2′,5′-dideoxyadenosine (3 and 15 μM), a selective inhibitor of adenylate cyclase. In conclusion, the FSH-induced Gαh/PLC-δ1 pathway-dependent Ca^{2+} influx of rat SCs is mediated by T-type Ca^{2+} channels and independent of in-store calcium release. (*Endocrinology* 149: 1031–1037, 2008)

UPON INTERACTING WITH its receptor on Sertoli cells (SCs), FSH activates the Gs/adenylate cyclase signaling pathway to elevate intracellular cAMP levels (1). On the other hand, FSH also induces an immediate Ca^{2+} influx through a Gs- and adenylate cyclase-independent phospholipase C (PLC)-dependent signaling pathway in rat SCs (2, 3). Recently, we have identified a Gαh/PLC-δ1 signaling pathway mediating FSH-induced immediate Ca^{2+} influx in rat SCs (4). However, the type of Ca^{2+} channel involved remains unknown.

Voltage-gated Ca^{2+} channels on plasma membrane are divided into T-, L-, N-, P/Q-, and R-type Ca^{2+} channels depending on their physiological and pharmacological properties (5). T-type Ca^{2+} channels are transient low-voltage-activated Ca^{2+} channels that control Ca^{2+} influx in excitable cells during small depolarizations around resting potential (6, 7). They are selectively blocked by Ni^{2+} and mibefradil in various cell types (8–10). L-type Ca^{2+} channels are long-term high-voltage-activated Ca^{2+} channels that are mainly found in muscle and endocrine cells where they initiate contraction and secretion (7, 11). They are blocked by the organic antagonists, including dihydropyridines, phenyl-akylamines, and benzothiazepines (12). N-type, P/Q-type, and R-type Ca^{2+} channels also require strong depolarization for activation (7, 13–15). They are unaffected by L-type Ca^{2+} antagonists but blocked by specific polypeptide toxins from snail and spider venoms, such as ω-conotoxin GVIA (16, 17), ω-agatoxin IVA (18, 19), and ω-conotoxin MVIIC (20, 21). They are expressed primarily in neurons of most fast synapses, where they initiate neurotransmission (13, 14).

Previous studies demonstrated that both low-voltage-activated and high-voltage-activated Ca^{2+} channels exist in rat SCs (22–25). Electrophysiological studies demonstrated that T-type Ca^{2+} channels of excitable cells are located in the membrane of immature SCs in primary cultures. These channels do not appear directly sensitive to FSH (24). On the other hand, the inhibition of N-type Ca^{2+} current by ω-conotoxin GVIA was shown to reduce about 50–60% of the FSH-induced protein secretion by rat SCs after 6 h of FSH treatment (25). However, no additional evidence has verified that N-type Ca^{2+} channels or the others mediate the FSH-induced immediate Ca^{2+} influx in rat SCs. Therefore, the present study was intended to identify the type of Ca^{2+} channel

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Abbreviations: DMSO, Dimethylsulfoxide; IP3, inositol 1,4,5-trisphosphate; PLC, phospholipase C; SC, Sertoli cell.

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involved in the FSH-induced immediate Ca^{2+} influx in rat SCs. Furthermore, the phenomenon that the activation of PLC leads to the generation of inositol 1,4,5-trisphosphate (IP3), which in turn induces a slight in-store Ca^{2+} release. That the in-store Ca^{2+} release is a prerequisite to the subsequent Ca^{2+} uptake through the Ca^{2+} channel is so-called capacitative Ca^{2+} influx (26, 27). Capacitative Ca^{2+} influx in rat SCs has been reported previously (28). Consequently, whether the FSH-induced IP3-dependent immediate Ca^{2+} influx (4) resulted from capacitative Ca^{2+} influx would also be verified.

Materials and Methods

Materials

Recombinant human FSH was from the National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance, CA). Nifedipine and mibefradil were purchased from Calbiochem (Merck Biosciences, Darmstadt, Germany). Collagenase, hyaluronidase, fura-2-AM, and poly-L-lysine were purchased from Sigma-Aldrich (St. Louis, MO). Myristoylated synthetic peptide of PLC-δ1 myr-TIPWNSLKQGYRHVHLL was synthesized by Synpep Corp. (Dublin, CA).

Animals

Wistar rats, 21 d old, from the Animal Facility of National Taiwan University were killed in a CO_2 chamber according to the National Institutes of Health Guidelines. Permission for using rodents for this study was approved by the Institutional Animal Care and Use Committee of Taipei Medical University (LAC-95-0067).

Cell culture

SCs were isolated from testes of 21-d-old rats as described previously (29). The cells from collagenase/hyaluronidase-treated testicular tubules were plated in 100-mm Corning culture dishes in DMEM/Ham's F-12 medium (DFM) (equal parts of DMEM and Ham's F-12 containing 100 μg/ml streptomycin, 100 U/ml penicillin, and 5 μg/ml fungizone) (all were purchased from Invitrogen, Carlsbad, CA) and cultured at 34 C in a humidified atmosphere with 5% CO_2. The cells were cultured in DFM for 3 d to allow a firm attachment of SCs. The SCs were subsequently cultured in DFM-6F media (DFM plus insulin, 1.0 μg/ml; epidermal grouth factor, 10 ng/ml; vitamin A and E, 200 ng/ml each; progesterone and hydrocortisone, 10^{-8} mol/liter each) as described previously (29) for an additional 2–3 d and then used for the study of the effect on FSH.

Measurement of intracellular calcium

SCs were cultured in six-well culture plates containing 9 × 22 mm poly-L-lysine-coated cover slides. The FSH-induced SC Ca^{2+} influx was determined after pretreating the cells with fura-2-AM (2 μM) as described in the previous study (4). The analysis of intracellular calcium changes was performed in a Hitachi F-4500 fluorescence spectrophotometer using an intracellular cation measurement system (Hitachi Scientific Instruments, Gaithersburg, MD).

Statistical analysis

Each data point represents the mean of three independent experiments and is presented as mean ± SEM. The statistical analysis was performed by one-way ANOVA and Duncan's multiple range test.

Results

Characterization of FSH-induced Ca^{2+} influx in rat SCs

To verify whether intracellular Ca^{2+} release participates in the FSH-induced immediate elevation of intracellular Ca^{2+} levels $[Ca^{2+}]_i$, dantrolene was used to block the intracellular Ca^{2+} release from endoplasmic reticulum stores. The pretreatment of rat SCs with dantrolene (25 μM), compared with control (Fig. 1A), did not affect the FSH-induced elevation of $[Ca^{2+}]_i$ (Fig. 1B). However, the administration of EGTA (0.2 mM) immediately abolished the FSH-induced rat SC intracellular Ca^{2+} elevation (Fig. 1C). These results are in accordance with our previous report that FSH induces an immediately inward Ca^{2+} mobilization in rat SCs within 100 sec under confocal microscopic observation (4).

To determine whether the FSH-induced SC immediate Ca^{2+} influx is elicited through capacitative Ca^{2+}-entry model (28), the elevation of $[Ca^{2+}]_i$ was performed in the absence of extracellular Ca^{2+}. Without extracellular Ca^{2+}, FSH (300 ng/ml) failed to elicit an intracellular Ca^{2+} elevation in SCs (Fig. 2A). However, thapsigargin (1 μM), an inhibitor of sarco-/endoplasmic reticulum ATPase, transiently evoked a 1.5-fold elevation of $[Ca^{2+}]_i$ in the SCs and then returned to basal level by a few minutes in the absence of extracellular Ca^{2+} (Fig. 2B). In these experimental conditions, the addition of extracellular Ca^{2+} (2.5 mM) caused a rapid rise in $[Ca^{2+}]_i$ (Fig. 2, A and B). On the other hand, the treatment of SCs with 0.1% BSA/PBS (Fig. 2C) or 0.1% dimethylsulfoxide (DMSO) (Fig. 2D), the solvents of FSH and thapsigargin stock solution,

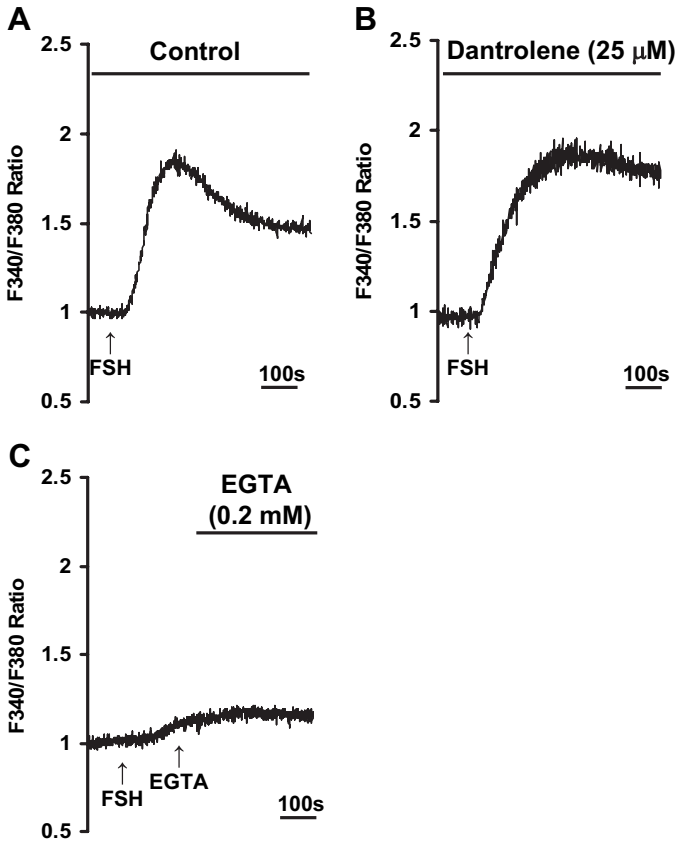

FIG. 1. The characterization of FSH-induced immediate Ca^{2+} elevation in rat SCs. Rat SCs were preincubated with fura-2-AM (5 μM) for 40 min at 34 C and then subjected to intracellular Ca^{2+} determination. A and B, FSH (300 ng/ml) was added to the incubation medium at the indicated time point in the absence (A) or presence (B) of dantrolene (25 μM); C, EGTA (0.2 mM) was added to medium to chelate extracellular calcium at the indicated time point after the administration of FSH.

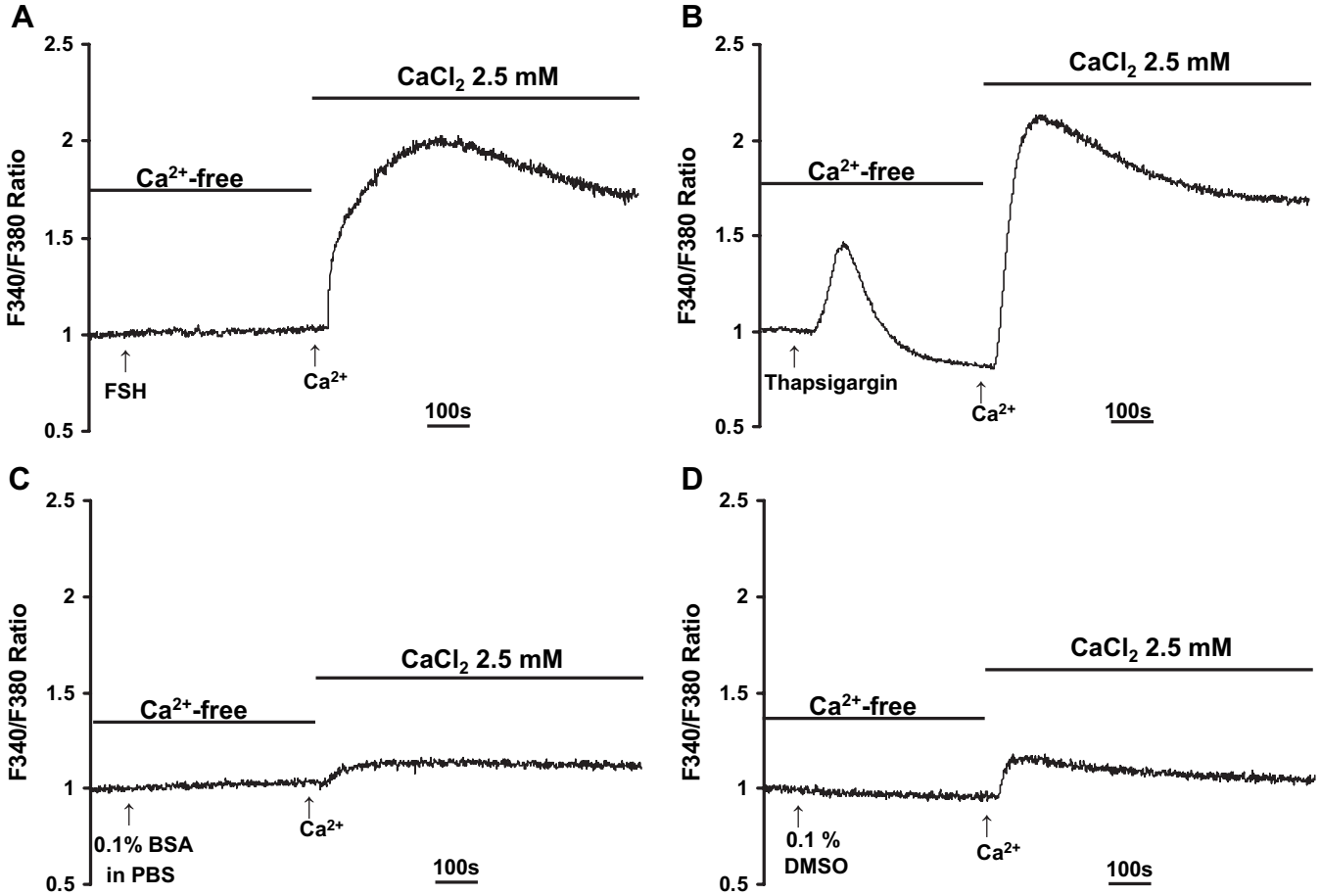

FIG. 2. The characterization of FSH-induced noncapacitative Ca^{2+} influx in rat SCs. Rat SCs were preincubated with fura-2-AM (5 μM) as stated in Fig. 1 before intracellular Ca^{2+} determination. The following were added at the indicated time points to the incubation medium without extracellular Ca^{2+}: A, FSH (300 ng/ml); B, thapsigargin (1 μM); C, BSA (0.1% dilution in PBS); or D, DMSO (0.1%). $CaCl_2$ (2.5 mM) was added to each incubation medium at the indicated time points. The 0.1% BSA and 0.1% DMSO were used as solvent control of FSH and thapsigargin, respectively.

respectively, did not evoke an elevation of $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}. However, supplementing with Ca^{2+} in these experimental conditions slightly elevated $[Ca^{2+}]_i$ (Fig. 2, C and D). These results suggest that the FSH-induced rat SC Ca^{2+} influx did not result from capacitative Ca^{2+} entry.

Type of Ca^{2+} channel associated with FSH-induced noncapacitative Ca^{2+} influx in rat SCs

The previous electrophysiological study on Ca^{2+} current of rat SCs by patch-clamp technique demonstrated the presence of a voltage-dependent T-type Ca^{2+} channel on plasma membrane of rat SCs (24). To further identify whether the FSH-induced immediate Ca^{2+} influx of rat SCs is mediated by T-type Ca^{2+} channels, $NiCl_2$ (10 μM), a selective blocker of T-type Ca^{2+} channels (<50 μM) (10), was included in the medium before the addition of FSH (300 ng/ml). As shown in Fig. 3, A and B, FSH failed to evoke Ca^{2+} influx in the presence of 10 μM $NiCl_2$. In addition, the pretreatment of rat SCs with mibefradil, also a specific inhibitor of T-type Ca^{2+} channels (9, 30), reduced the FSH-induced Ca^{2+} influx dose dependently (Fig. 3C). Mibefradil at 10 and 100 μM exhibited a significant ($P < 0.05$) inhibitory effect on FSH-induced rat SC Ca^{2+} influx by 50 and 90%, respectively (Fig. 3D). On the other hand, the pretreatment with nifedipine (10 and 50 μM) and ω-conotoxin (100 and 500 nM) to specifically inhibit the activities of L- and N-type Ca^{2+} channels, respectively, did not affect the FSH-induced rat SC Ca^{2+} influx (Fig. 4, A and B).

The roles of Gs/adenylate cyclase- and Gαh/PLC-δ1-signaling in FSH-induced rat SC T-type Ca^{2+}-channel-mediated Ca^{2+} influx

FSH-induced rat SC Ca^{2+} influx was independent of the Gs/adenylate cyclase pathway (3). In our previous study (4), 2′,5′-dideoxyadenosine (3 and 15 μM) was used to inactivate adenylate cyclase and resulted in a dose-dependent reduction in the FSH-induced accumulation of intracellular cAMP. However, the pretreatment of rat SCs with 2′,5′-dideoxyadenosine (3 and 15 μM) did not suppress but slightly enhanced the FSH-induced rat SC Ca^{2+} influx (Fig. 5A) in the present study. Furthermore, pretreatment of rat SCs with U73122 (5 μM), a specific inhibitor of phosphatidylinositol-dependent PLCs, or with myristoylated PLC-δ1 peptide (1 μM) to competitively inhibit the activation of PLC-δ1 by Gαh

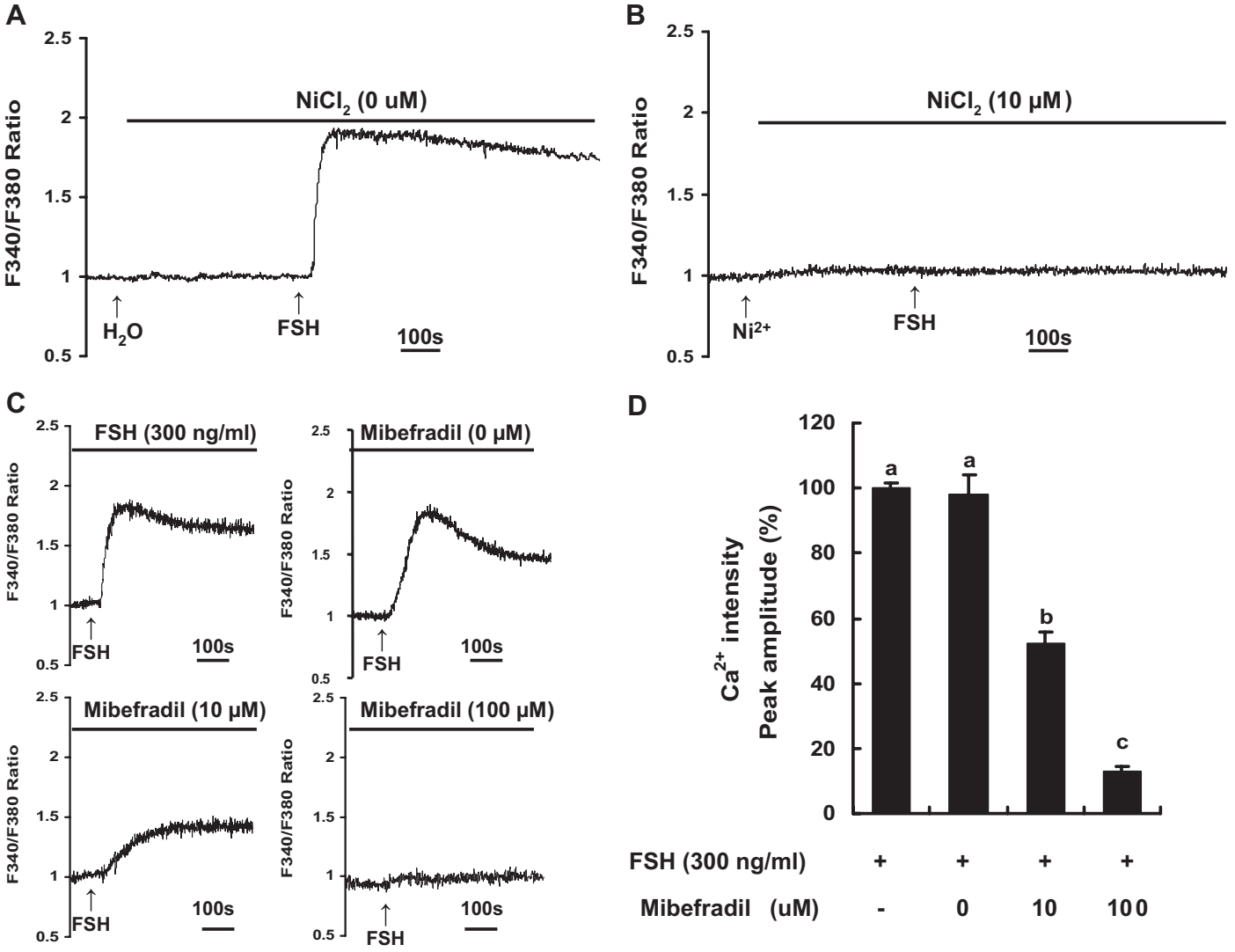

FIG. 3. The effect of $NiCl_2$ or mibefradil on FSH-induced Ca^{2+} influx in rat SCs. Rat SCs were preincubated with fura-2-AM (5 μM) as described above. A and B, H_2O (as solvent control of $NiCl_2$) (A) or $NiCl_2$ (10 μM) (B) was added into the incubation medium at the indicated time points. After 500 sec incubation, FSH (300 ng/ml) was added into the incubation medium. C, Similarly, SCs were preincubated with fura-2-AM (5 μM) with or without mibefradil [0 (H_2O, as control), 10, or 100 μM] for 40 min at 34 C and then subjected to intracellular Ca^{2+} determination. FSH (300 ng/ml) was added to the incubation medium at the indicated time points. D, The data from three independent experiments of various mibefradil treatments were analyzed by one-way ANOVA and Duncan multiple range test. Data shown in D represent means ± SEM (n = 3). *Different letters above the columns* indicate significant differences between the means ($P < 0.05$). H_2O was used as solvent control of mibefradil.

also significantly ($P < 0.05$) reduced the FSH-induced Ca^{2+} influx (Fig. 5B). These results indicate an essential role of the Gαh/PLC-δ1 signaling pathway in this event. On the other hand, pretreatment of SCs with DMSO (0.1%), as a solvent control of U73122 or myristic acid (1 μM), did not affect the FSH-induced Ca^{2+} influx (Fig. 5B), thus excluding the off-target effect of U73122 or myristoylated PLC-δ1 peptide, respectively.

Discussion

Our previous study demonstrated that FSH elicits a novel Gαh/PLC-δ1 signaling pathway, distinct from the classical Gs/adenylate cyclase pathway, which induces intracellular IP3 generation and evokes extracellular Ca^{2+} influx in rat SCs (4). Although the phenomenon of FSH-induced immediate rat SC Ca^{2+} influx was documented in other studies (3, 31–33), as to which type of Ca^{2+} channel was involved in this event remains unclear. In the present study, our results define that T-type Ca^{2+} channels are responsible for FSH-induced immediate Ca^{2+} influx in rat SCs. Our results also reveal that FSH-induced T-type Ca^{2+} current in rat SCs is independent of the mode of capacitative Ca^{2+} entry.

The activation of the PLC pathway results in the generation of intracellular IP3, which stimulates the release of Ca^{2+} store from the endoplasmic reticulum (34). This release of in-store Ca^{2+} is generally associated with the subsequent activation of calcium channels and leads to an increase in the Ca^{2+} entry across the plasma membrane. The elevated intracellular Ca^{2+} levels either replenish the in-store calcium or contribute to Ca^{2+}-dependent signaling. This process of Ca^{2+} entry is called capacitative Ca^{2+} entry or store-operated Ca^{2+} entry (26, 27). Rossato *et al.* (28) reported that the depletion

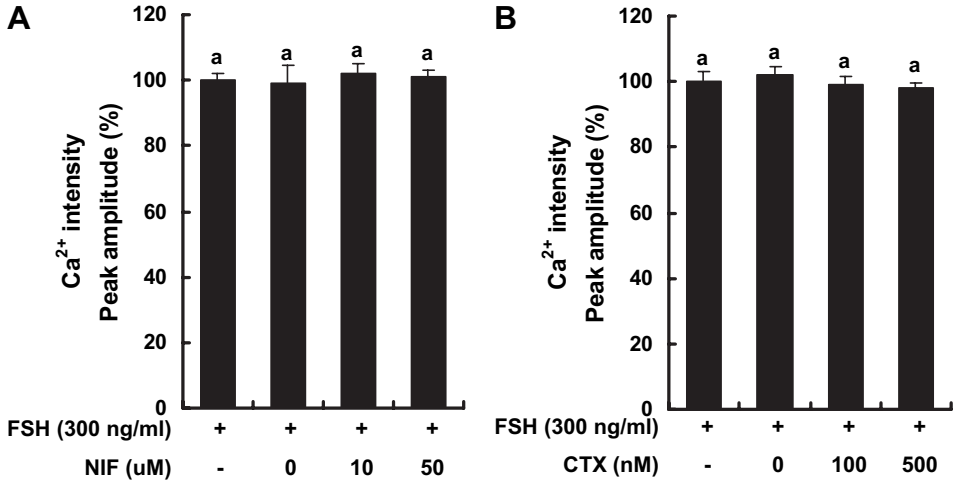

FIG. 4. The effects of nifedipine and ω-conotoxin on the FSH-induced Ca^{2+} influx in rat SCs. In addition to the pretreatment with fura-2-AM (5 μM), rat SCs simultaneously pretreated with or without nifedipine [0 (H_2O, as control), 10, or 50 μM] (A) or ω-conotoxin [0 (0.1% DMSO, as solvent control), 100, or 500 nM] (B). Peak amplitudes of intracellular Ca^{2+} level in response to FSH treatment were obtained from three independent experiments and analyzed by one-way ANOVA and Duncan multiple range test. Data represent means ± SEM (n = 3). *Different letters above the columns* indicate significant differences between the means ($P < 0.05$). H_2O and 0.1% DMSO was used as solvent controls of nifedipine and ω-conotoxin, respectively.

of internal Ca^{2+} stores by thapsigargin, an inhibitor of sarco-/endoplasmic reticulum ATPase, induces Ca^{2+} influx from the extracellular medium in rat SCs, providing evidence for the existence of capacitative Ca^{2+} entry in these cells. This view was supported by the observation in Fig. 2B of the present study. Although our previous study demonstrated that the interaction of FSH with its receptor on rat SCs induces intracellular IP3 generation (4), the FSH-induced immediate rat SC Ca^{2+} influx was not caused by the IP3-dependent capacitative Ca^{2+} entry. This claim was supported by evidence that FSH failed to evoke the elevation of $[Ca^{2+}]_i$ in the absence of external Ca^{2+} and that FSH induced Ca^{2+} influx after the pretreatment of SCs with dantrolene, an inhibitor of intracellular Ca^{2+} release. Furthermore, pretreatment with U73122 or synthetic myristoylated PLC-δ1 peptide to inhibit the activity of PLC-δ1 resulted in the reduction of FSH-induced intracellular IP3 generation (4) and Ca^{2+} influx in rat SCs. These results indicate that FSH-induced Ca^{2+} influx is mediated by intracellular IP3-dependent signaling but not through capacitative Ca^{2+} entry in rat SCs.

The recent identification of IP3 receptors on plasma membrane and the association of those receptors with Ca^{2+} channels (35–37) may unveil the mechanism of FSH-induced IP3-dependent, noncapacitative Ca^{2+} entry in rat SCs. Further efforts are required to demonstrate the existence of IP3 receptors on rat SC plasma membrane. Consequently, the direct action of IP3 in the activation of transient Ca^{2+} channels needs to be further substantiated.

In addition to T-type Ca^{2+} channels, L-, N-, and P/Q-type Ca^{2+} channels were also found on rat SC plasma membrane

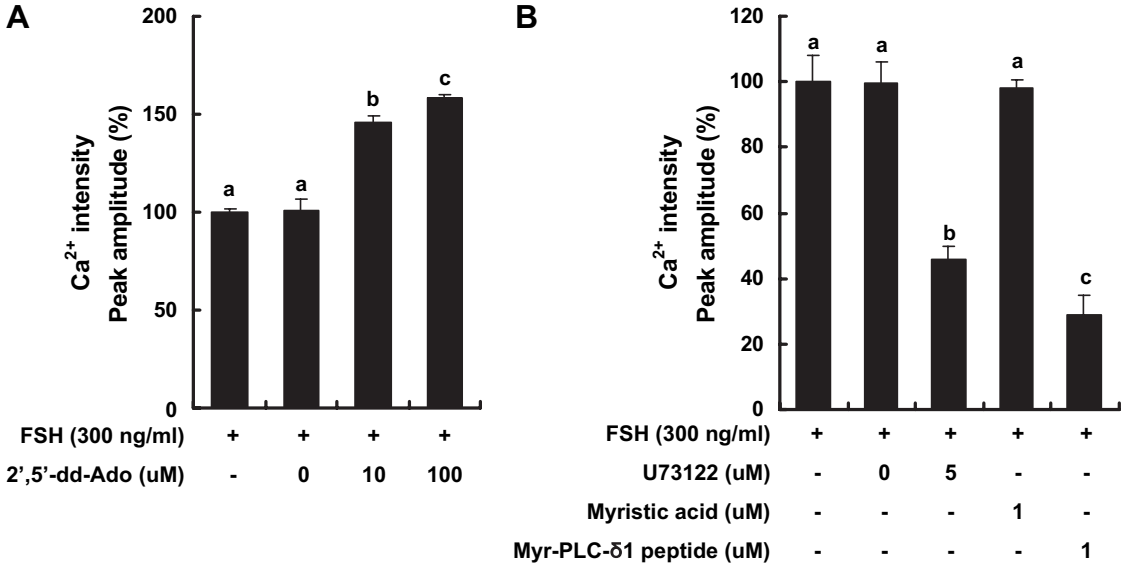

FIG. 5. The effect of 2′,5′-dideoxyadenosine (2′,5′-dd-Ado) on the FSH-induced Ca^{2+} influx in rat SCs. A, Rat SCs were preincubated with fura-2-AM (5 μM) and with or without 2′,5′-dd-Ado [0 (H_2O, as control), 10, or 100 μM] for 40 min at 34 C and then subjected to intracellular Ca^{2+} determination. B, In addition to the pretreatment with fura-2-AM (5 μM), rat SCs were simultaneously pretreated with or without U73122 [0 (0.1% DMSO) and 5 μM], myristic acid (1 μM), or myristoylated PLC-δ1 synthetic peptide (myr- PLC-δ1 peptide) (1 μM). Peak amplitudes of intracellular Ca^{2+} levels in response to FSH treatments were obtained from three independent experiments and analyzed by one-way ANOVA and Duncan multiple range test. Data represent means ± SEM (n = 3). *Different letters above the columns* indicate significant differences between the means ($P < 0.05$). DMSO (0.1%) was used as solvent control of U73122. Myristic acid was used as control for the off-target effect of myristoylated PLC-δ1 synthetic peptide.

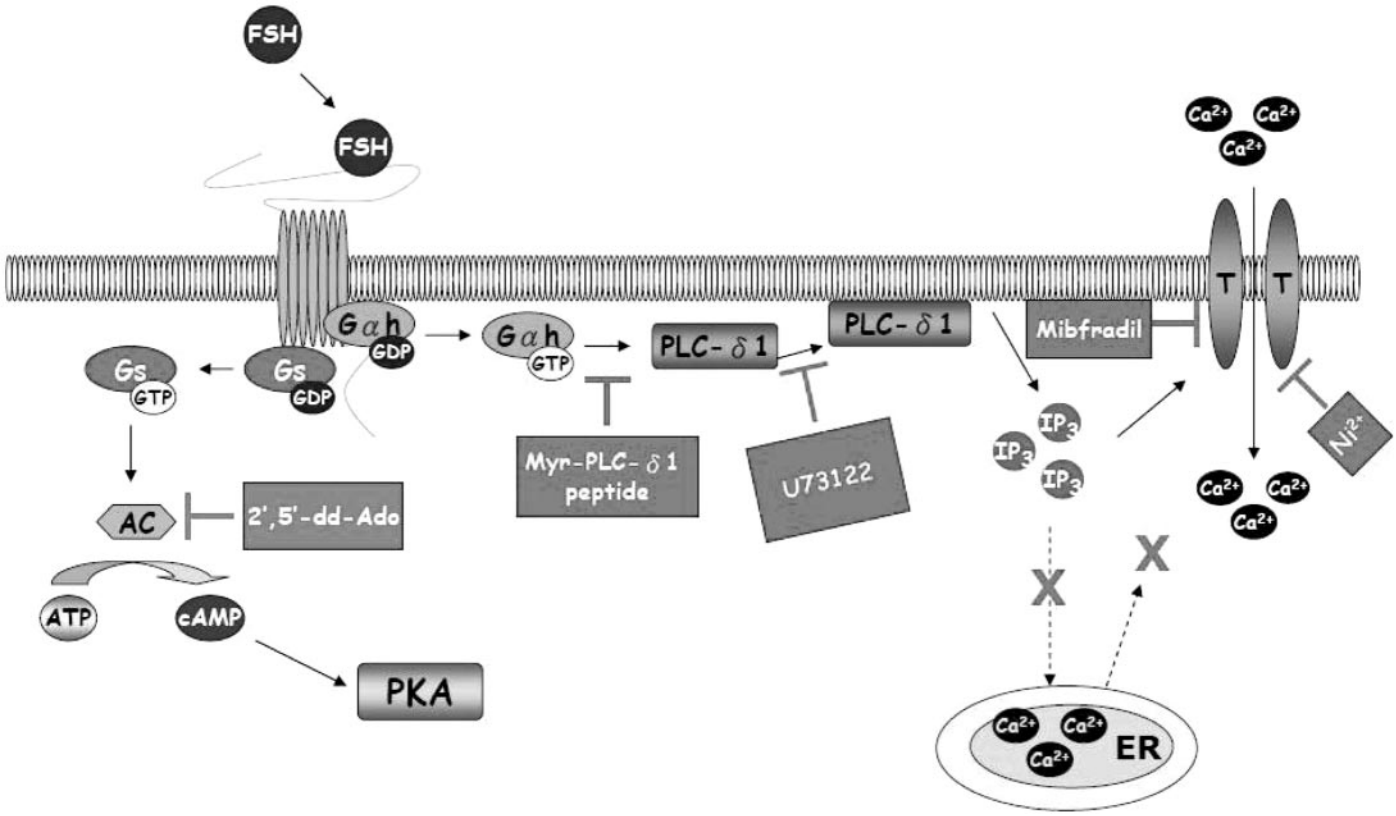

FIG. 6. Schematic diagram summarizes signaling pathways of FSH-induced Ca^{2+}-channel activation and calcium influx. The T-type Ca^{2+} channel is activated directly by IP3 instead of capacitative Ca^{2+} entry through IP3-induced in-store Ca^{2+} release. AC, Adenylate cyclase; 2′,5′-dd-Ado, 2′,5′-dideoxyadenosine; ER, endoplasmic reticulum; PKA, protein kinase A.

(22–25). However, this study demonstrates that T-type Ca^{2+} channels are essential for the FSH-induced immediate Ca^{2+} influx in rat SCs. $NiCl_2$ (10 μM) was shown to block the membrane depolarization-induced T-type Ca^{2+} current in rat SCs (24). The similar inhibitory effect of $NiCl_2$ (10 μM) was observed in the FSH-induced rat SC Ca^{2+} influx in the present study. In addition, mibefradil, a T-type Ca^{2+}-channel antagonist, dose-dependently reduced the FSH-induced rat SC Ca^{2+} influx. These data indicate a critical role of T-type Ca^{2+} channels in the FSH-induced immediate Ca^{2+} influx of SCs.

The pretreatment of rat SCs with ω-conotoxin (4 μM) caused a 50–60% reduction of FSH-induced protein secretion after 6 h of treatment (25). At high concentrations (>1 μM), ω-conotoxin suppressed the activities of both L- and T-type Ca^{2+} channels in neuronal cells (38) and rat SCs (23, 24), whereas ω-conotoxin at low concentration blocked N-type Ca^{2+} channels. In this study, a low dose of ω-conotoxin (100 and 500 nM) did not affect the FSH-induced rat SC Ca^{2+} influx. Similarly, the FSH-induced rat SC Ca^{2+} influx was not affected by the L-type Ca^{2+}-channel inhibitor nifedipine. All the above findings indicate that the T-type Ca^{2+} channel plays a crucial role in mediating the FSH-induced immediate Ca^{2+} influx in rat SCs.

FSH-induced immediate Ca^{2+} influx in rat Sertoli cells has been well demonstrated in our laboratories and others. Our previous study identified the involvement of the Gαh/PLC-δ1signaling pathway in this event (4). According to our previous study (4), the occurrence of the Gαh/PLC-δ1 signaling pathway is more prominent at a FSH level greater than 300 IU/liter (3 ng/ml). This might imply that the activation of Gαh/PLC-δ1 signaling is associated with the feedback mechanism of SCs, through releasing inhibin, at elevated circulating FSH level (25). This speculation needs to be further substantiated. In conclusion, as summarized in the scheme presented in Fig. 6, the data in this study further demonstrate that FSH-induced Gαh/PLC-δ1-dependent Ca^{2+} influx in rat SCs is mediated by T-type Ca^{2+} channels. It is activated independent of in-store calcium release, via a noncapacitative calcium-entry model.

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Disclosure Statement: The authors have nothing to disclose.

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