

Serotonin stimulates GnRH secretion through the c-Src-PLC γ 1 pathway in GT1–7 hypothalamic cells

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Abstract

Serotonin is a neurotransmitter that alters the hypothalamic-pituitary-adrenal axis. To date, however, the molecular mechanisms underlying the role of serotonin in hormone secretion have remained largely unclear. In this study, we report that serotonin activates phospholipase C (PLC) γ 1 in an Src-dependent manner in hypothalamic GT1–7 cells, and that pretreatment with either 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazole [3, 4-*d*] pyrimidine, an Src-kinase inhibitor, or U73122, a PLC inhibitor, attenuates the serotonin-induced increase in calcium levels. Also, PLC γ 1 binds to c-Src through the Src-homology (SH) 223 domain upon serotonin treatment. Moreover, calcium increase is alleviated in the cells

transiently expressing SH223 domain-deleted PLC γ 1 or lipase inactive mutant PLC γ 1, as compared with cells transfected with wild-type PLC γ 1. Furthermore, the inhibition of the activities of either PLC or Src results in a significant diminution of the serotonin-induced release of gonadotropin-releasing hormone (GnRH). In addition, the results of our small-interfering RNA experiment confirm that endogenous PLC γ 1 is a prerequisite for serotonin-mediated signaling pathways. Taken together, our findings demonstrate that serotonin stimulates the release of GnRH through the Src-PLC γ 1 pathway, via the modulation of intracellular calcium levels.

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Introduction

Serotonin is a neurotransmitter that plays a variety of roles in the central nervous system. In a previous study, the functional relationship between serotonin and the neurohypophysis system was described at the level of the anterior hypothalamus (Ferris *et al.* 1997). Serotonergic nerve fibers and receptors have been detected in both the supraoptic nucleus and the paraventricular nucleus, in which they appear to contribute to the regulation of adeno- and neurohypophysial hormones (Sawchenko *et al.* 1983, Chaouloff 2000, Kang *et al.* 2004). These findings indicate that serotonin may function as a mediator of hormone secretion.

Gonadotropin-releasing hormone (GnRH) release is essential for the maintenance of gonadotropin secretion and normal reproductive function. The secretion of GnRH is modulated by neurotransmitters (Moguilevsky & Wuttke 2001), catecholamines (Becu-Villalobos & Libertun 1995), and neuropeptides (Woller *et al.* 1992). Within the hypothalamus, the auto-regulatory control of GnRH neural activity is integrated with other hormonal inputs to provide a more complex control system. Several mechanisms for the

generation of GnRH have been suggested, including the bursts of action potentials (Kusano *et al.* 1995) and cAMP-dependent cation channel activations (Sakakibara *et al.* 1998).

Phospholipase C (PLC) γ 1 possesses two Src-homology 2 (SH2) domains and one SH3 domain, between the X- and Y-catalytic domains (Stahl *et al.* 1988, Suh *et al.* 1988). The SH domains have been determined to perform a vital function in the PLC γ 1-mediated signaling pathway (Chang *et al.* 1999). The SH2 domains of PLC γ 1 have been implicated in the association between PLC γ 1 and the activated receptor tyrosine kinases, and the SH3 domain has been associated with the mitogenic effects of PLC γ 1 (Huang *et al.* 1995, Smith *et al.* 1996).

Following earlier reports that implicated serotonin in the development and migration of GnRH neurons (Pronina *et al.* 2003), a great deal of attention has become focused on the role of serotonin in GnRH release (Moguilevsky & Wuttke 2001, Wada *et al.* 2006). The demonstration of synaptic contacts between the serotonergic system and the GnRH neurons suggested that serotonin could act directly on GnRH release. However, little knowledge is available at present with regard to the molecular mechanisms inherent to the role of serotonin.

In this study, we investigated the effects of serotonin on hypothalamic GT1-7 cells, in order to gain understanding into the molecular mechanisms underlying serotonin-mediated hormone secretion. We have determined that serotonin activates PLC γ 1 in the hypothalamic GT1-7 cells, and further demonstrated that the activities of PLC and Src affect GnRH secretion. These findings provide novel insights into the manner in which PLC γ 1 contributes to the serotonin-mediated release of GnRH.

Materials and Methods

Reagents

Phospho-mitogen-activated protein kinase kinase (MEK)1/2 (Ser²¹⁷/Ser²²¹), phospho-extracellular signal-regulated kinase (ERK)1/2 (Thr²⁰²/Tyr²⁰⁴), MEK1/2, and ERK1/2 antibodies were purchased from Cell Signaling Technology (New England Biolabs, Beverly, MA, USA) and horseradish peroxidase-conjugated secondary antibodies were obtained from Kirkegaard and Perry Lab. (Gaithersburg, MD, USA). PLC γ 1, phospho-c-Src, c-Src, and actin antibodies were purchased from Sigma-Aldrich. Serotonin, U73122, and 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazole [3, 4-*d*] pyrimidine (PP2) were obtained from Calbiochem (San Diego, CA, USA).

Cell culture

GT1-7 hypothalamus cells, which were donated by Dr Richard Weiner (University of California, San Francisco, CA, USA), were grown in a culture medium consisting of 500 ml Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY, USA), containing 0.584 g/l L-glutamate and 4.5 g/l glucose, mixed with 500 ml F-12 medium containing 0.146 g/l L-glutamate, 1.8 g/l glucose, 100 μ g/ml gentamicin, 2.5 g/l sodium carbonate, and 10% heat-inactivated fetal bovine serum.

Immunoprecipitation

The cells, after serotonin stimulation with or without inhibitors, were placed on ice, washed twice in ice-cold PBS, and lysed in lysis buffer containing 50 mM Tris (pH 8.0), 100 mM NaCl, 20 mM NaF, 10 mM sodium pyrophosphate, 5 mM EDTA, 1% Nonidet P-40, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 10 μ g/ml soybean trypsin inhibitor, 10 μ g/ml pepstatin, and 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride. The solubilized lysates were then clarified via 10 min centrifugation at 8000 g, pre-cleared with agarose, and incubated with specific antibodies and protein-A agarose. The immunoprecipitates were collected and washed four times in lysis buffer. After 5-min heating at 95 °C, the samples were centrifuged, and the supernatants were analyzed on SDS-PAGE (8–16%) gradient gels.

Immunoblot analysis

The cells were grown on six-well plates and serum-starved for 24 h prior to treatment with the indicated agents. The media were aspirated, and the cells were washed twice in ice-cold PBS and lysed in 100 μ l lysis buffer. The samples were then briefly sonicated, heated for 5 min at 95 °C, and centrifuged for 5 min. The supernatants were electrophoresed on SDS-PAGE (8–16%) gradient gels, and transferred to polyvinylidene difluoride membranes. The blots were incubated overnight at 4 °C with primary antibodies, then washed six times in Tris-buffered saline/0.1% Tween 20 (Sigma Aldrich) prior to 1 h probing with horseradish peroxidase-conjugated secondary antibodies at room temperature. The blots were then visualized via enhanced chemiluminescence (Amersham Biosciences). In some cases, the blots were stripped and reprobed using other antibodies.

Transient transfection

To construct and express cDNA encoding whole and mutant forms of PLC γ 1, rat PLC γ 1 cDNA was N-terminally tagged with FLAG sequences and sub-cloned into FLAG-cyto-megalovirus (CMV) (Bae *et al.* 1998). Src wild-type and dominant-negative genes were provided by Dr Kitagawa (Kitagawa *et al.* 2002). The GT1-7 cells were transfected with FLAG-wild-type PLC γ 1, FLAG-lipase inactive mutant (LIM) PLC γ 1, and FLAG-SH223 domain-deleted PLC γ 1, using LipofectAMINE (Invitrogen). After 5 h, the cells were switched to serum-containing medium for 24 h, followed by replacement with serum-free medium overnight. The cells were then treated with agonists and collected in lysis buffer for immunoblot analysis and immunoprecipitation. DNA transfection was performed using LipofectAMINE, in accordance with the manufacturer's instructions.

Calcium measurement

Sub-confluent GT1-7 cells cultured in 175-cm² flasks were collected via trypsinization (0.05% trypsin and 0.02% EDTA) and centrifuged (230 g, 5 min). The cells were then suspended in culture medium at a final concentration of 5×10^6 cells/ml, and incubated for 20 min with fura 2-acetoxymethyl (AM; 10 μ M) and 0.02% pluronic F-127 at 30 °C, with continuous agitation. At the end of the incubation, the cells were centrifuged (230 g, 5 min) and washed in culture medium. The cells were washed twice in Hanks' buffer (in mM: 137 NaCl, 5.4 KCl, 0.34 Na₂HPO₄, 5.5 glucose, 4.2 NaHCO₃, 0.8 MgSO₄, and 10 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES) Sigma Aldrich), with 0.1 mM EGTA for the first wash only, pH 7.4). The cells were resuspended in this buffer at a final concentration of 2.7×10^6 cells/ml, and maintained in darkness at 4 °C until use. Calcium concentrations were measured using an RF-5301PC Spectrofluorophotometry (Shimadzu, Kyoto, Japan), after 4-min incubation at 37 °C (excitation at 340 and 380 nm, emission

at 510 nm). The levels of free calcium in the cytosol were determined according to a previously described method (Grynkiewicz *et al.* 1985).

Small-interfering RNA (siRNA) for PLC γ 1

siRNA of PLC γ 1 (PLC γ 1 siRNA) was purchased from Dharmacon (Lafayette, CO, USA). The sequences of siRNA for luciferase (control siRNA) were as follows: sense, 5'-CUUACGCUGAGUACUUCGAdTdT-3'; antisense, 5'-UCGAAGUACUCAGCGUAAAGdTdT-3'. Double-strand RNA was produced using the conditions described previously (Patterson *et al.* 2002). siRNA was transfected into GT1-7 cells using LipofectAMINE.

GnRH measurement via the RIA method

Static incubation and GnRH RIA were conducted as previously described, with some modifications (Cho *et al.* 1998). In brief, the hypothalamic fragments were rapidly isolated from 10-week-old male Institute of Cancer Research (ICR) mice and cultured in 96-well tissue culture plates (one fragment per well) in 0.3 ml DMEM supplemented with an F12 nutrient mixture. The boundaries of the hypothalamic fragments were caudally the anterior border of the mammillary body, rostrally the apex of the isocles triangle-shaped region and laterally the lateral hypothalamic sulci. Hypothalamic fragments were sliced at a depth of approximately 1 mm. For equilibration, the tissues were pre-incubated for 1 h at 37 °C in a humidified incubator, under 5% CO₂ tension. After 1 h, the tissues were washed twice in PBS, and incubated for an additional 1 h in fresh medium with the appropriate vehicle or drugs. The media were then collected and centrifuged for 5 min at 10 000 g, in order to prevent any possible contamination of the cells. GnRH RIA was conducted using the Chen-Ramirez GnRH antiserum, CRR13B73 (generously provided by Dr V D Ramirez, University of Illinois, Urbana, IL, USA) at a final dilution of 1:180 000. Sensitivity at 80% binding was approximately 0.5 pg/tube. The intra- and interassay coefficients of variation were determined to be 4–5 and 5–10% respectively for a 5 pg dose of synthetic GnRH.

Band intensity analysis

The band intensity signal was quantified using Image Gauge software (version 3.12; Fujifilm).

Statistical analysis

Data were expressed as means \pm s.e.m. Statistical analyses were conducted using SigmaStat (SPSS, Inc., Chicago, IL, USA). The differences were considered to be significant at a *P* value of <0.05. We used one-way ANOVA with Holm–Sidak comparisons to determine the effects of PP2 and U73122 on

GnRH secretion, and used one-way ANOVA with a *post-hoc* Fisher's test in order to analyze band intensity.

Results

Serotonin phosphorylates PLC γ 1 in GT1-7 cells in a time-dependent manner

In order to determine the role of PLC γ 1 in serotonin signaling, we conducted an immunoprecipitation experiment to ascertain whether PLC γ 1 was activated by serotonin in the hypothalamic GT1-7 cells, which have been shown to express the serotonin receptor (Doucet *et al.* 1995). The cells were initially serum-starved for 24 h to remove any stimulants from the media, and were then treated with either vehicle alone or with serotonin (1 μ M) for the indicated times. The phosphorylation of endogenous PLC γ 1 increased after stimulation with serotonin (Fig. 1A). A previous report suggested that PLC γ 1 phosphorylation on Tyr⁷⁸³ residue was essential for the activity of PLC γ 1 (Kim *et al.* 1991). Therefore, we used a phospho-specific antibody, which could recognize the phosphotyrosine residue of PLC γ 1 (Tyr⁷⁸³) to detect tyrosine phosphorylated PLC γ 1 (Choi *et al.* 2005). Phosphorylation reached maximum levels at 10 min, and began to decrease at 30 min. Then, in order to ascertain whether the activation of PLC γ 1 could be correlated with the activation of downstream signaling molecules, we employed western blot analysis to determine whether serotonin induced the activation of MEK1/2 and ERK1/2. MEK1/2 and ERK1/2 were phosphorylated in a time-dependent manner upon serotonin stimulation, whereas total MEK1/2 and ERK1/2 levels remained unaffected (Fig. 1B). Taken together, these findings indicate that PLC γ 1 may play a role in serotonin signaling.

c-Src is involved in serotonin-mediated PLC γ 1 phosphorylation

PLC has been reported to function as a substrate for members of the Src-kinase family (Haendeler *et al.* 2003, Stope *et al.* 2004). It has also been demonstrated that c-Src plays a crucial role in serotonin-mediated calcium responses (Giusti *et al.* 1999, Tolloczko *et al.* 2002, Chang *et al.* 2004), thereby raising the possibility that c-Src may play some role in serotonin signaling. In order to better understand the signaling pathway involved in the serotonin-mediated activation of PLC γ 1, we pretreated the cells with PP2 (10 μ M), an Src-kinase inhibitor, prior to serotonin (1 μ M) administration, and then assessed PLC γ 1 phosphorylation via western blot analysis. The tyrosine phosphorylation of PLC γ 1 (Tyr⁷⁸³) was impaired in the presence of PP2 (Fig. 2A). Subsequently, we attempted to determine whether the activation of downstream signaling molecules was also affected by treatment with the Src-kinase inhibitor. The potency of MEK1/2 and ERK1/2 phosphorylation was attenuated substantially as a result of pretreatment with PP2 (Fig. 2B). For further confirmation, we conducted an

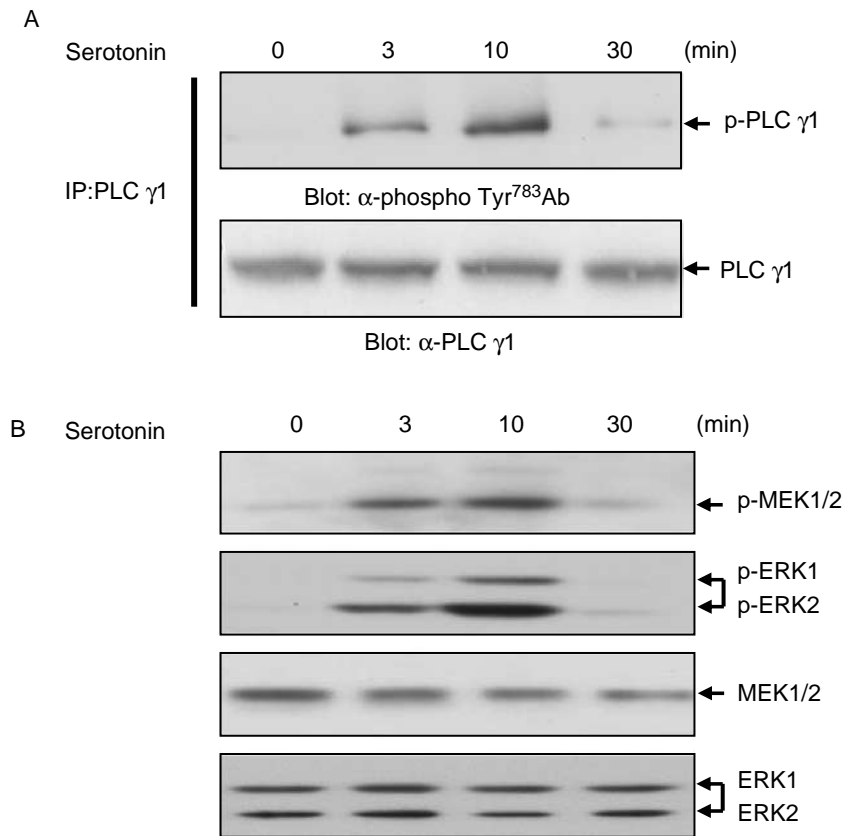


Figure 1 Serotonin phosphorylates PLC γ 1 in GT1-7 cells in a time-dependent manner. (A) The phosphorylation of PLC γ 1 upon serotonin stimulation. After 24-h starvation, the GT1-7 cells were treated with serotonin (1 μ M) for the indicated times. Immunoprecipitation (IP) with anti-PLC γ 1 antibody was performed, followed by blotting with phospho-specific antibody, which could recognize phosphotyrosine residue of PLC γ 1 (Tyr⁷⁸³), in order to monitor the phosphorylation of endogenous PLC γ 1. The lower panel demonstrates the equivalent loading of total PLC γ 1 in the whole cell lysates utilized in the immunoprecipitation reactions, using anti-PLC γ 1 antibody. (B) The activation of downstream signaling molecules upon stimulation with serotonin. After 24-h starvation, the cells were stimulated with serotonin (1 μ M) for the indicated times. Twenty micrograms of cell lysates were prepared and blotted with anti-phospho-MEK1/2 and extracellular signal-regulated kinase (ERK)1/2 antibodies. Blotting was conducted with anti-MEK1/2 and ERK1/2 antibodies as a control for protein loading. The results were representative of four independent experiments.

additional investigation into the effects of serotonin on the phosphorylation of c-Src. c-Src was phosphorylated directly upon stimulation with serotonin (Fig. 2C). In order to clarify the hierarchy inherent to Src and PLC γ 1, we have investigated the effects of an Src dominant-negative mutant on serotonin-mediated PLC γ 1 phosphorylation. PLC γ 1 phosphorylation (Tyr⁷⁸³) due to serotonin was completely suppressed in the Src mutant-transfected cells (Fig. 2D). To delineate, we have investigated the effects of U73122, a PLC inhibitor, on serotonin-induced mitogen-activated protein kinase (MAPK) activity. As MAPK activity was completely suppressed by U73122, PLC γ 1 appears to function as an upstream regulator of the MAPK pathway (Fig. 2E). Collectively, these results suggest that Src is involved in the serotonin-mediated activation of PLC γ 1.

PP2 and U73122 suppress the serotonin-induced calcium release

Calcium-mobilizing stimuli have been shown to control GnRH release within the hypothalamus (Senthilkumaran *et al.* 2001, Beauvillain & Prevot 2004). Thus, hypothalamic tissue or cell lines are considered to constitute a good model for the study of the relationship between calcium and hormone secretion. On the basis of our biochemical results, we hypothesized that the serotonin-mediated activation of PLC γ 1 might result in GnRH release via the modulation of intracellular calcium levels. First, in order to gain some insight into the role of PLC γ 1 in the process of hormone secretion, we pretreated the cells with U73122 (2 μ M) prior to serotonin (1 μ M) administration, and then measured calcium levels in the GT1-7 cells. Calcium levels were increased upon

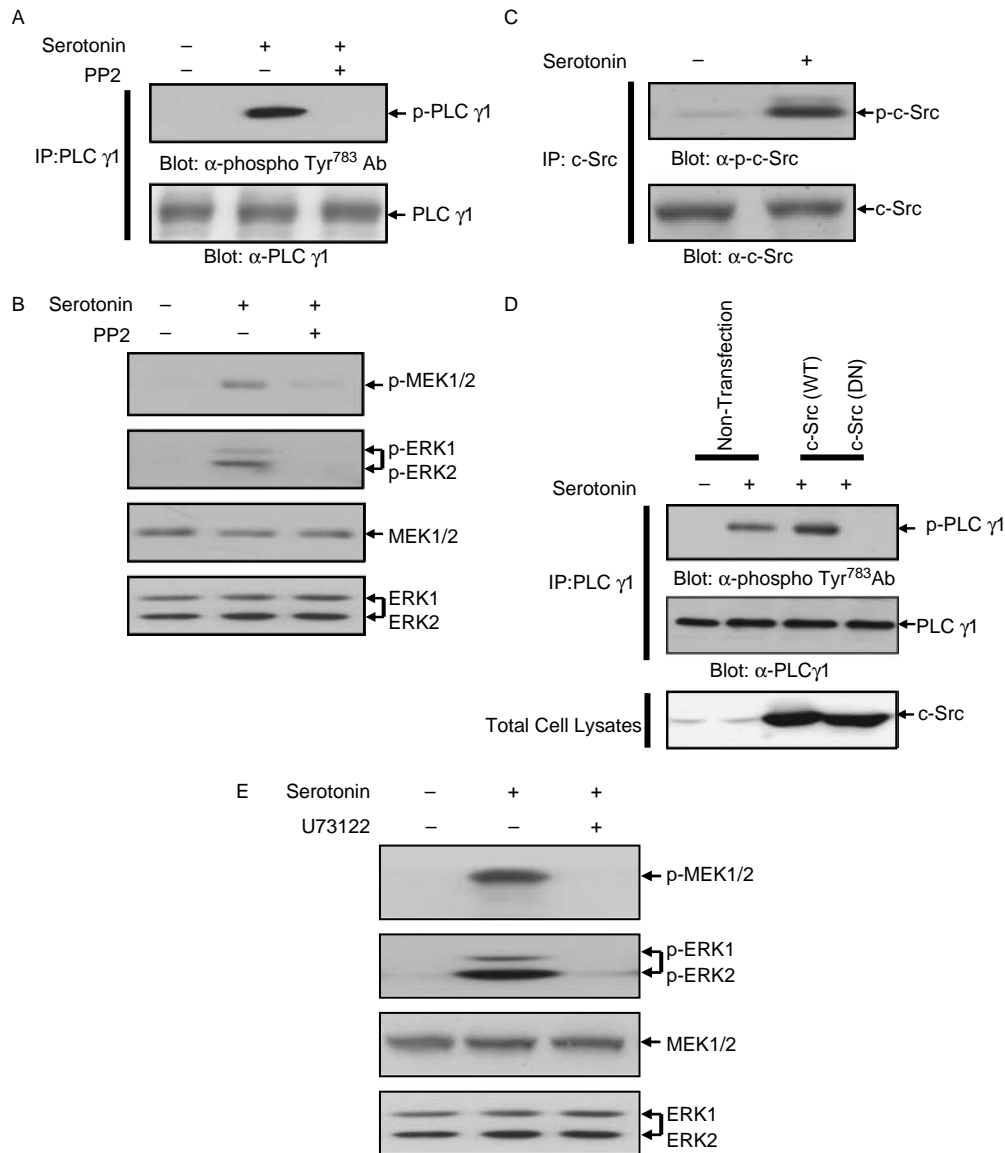


Figure 2 c-Src is involved in the serotonin-mediated PLC γ 1 phosphorylation. (A) The phosphorylation of PLC γ 1 by serotonin proceeds via Src kinase. The GT1-7 cells were stimulated for 10 min with serotonin (1 μ M) either in the absence or presence of PP2 (10 μ M). Immunoprecipitation (IP) with anti-PLC γ 1 antibody was performed, followed by blotting with phospho-specific antibody, which could recognize phosphotyrosine residue of PLC γ 1 (Tyr⁷⁸³), in order to monitor the phosphorylation of endogenous PLC γ 1. The lower panel demonstrates the equivalent loading of total PLC γ 1 in the whole cell lysates utilized in the immunoprecipitation reactions, using anti-PLC γ 1 antibody. (B) The phosphorylation of downstream signaling molecules depends on the activity of Src. After 24-h starvation, cells were stimulated with serotonin (1 μ M) for 10 min, either in the presence or absence of PP2 (10 μ M). The cell lysates were analyzed via western blotting for anti-phospho-MEK1/2 and ERK1/2 antibodies. Blotting with anti-MEK1/2 and ERK1/2 antibodies was conducted as a protein loading control. (C) The serotonin-induced phosphorylation of c-Src. After 24-h starvation, the cells were stimulated with serotonin (1 μ M) for 10 min. The c-Src was immunoprecipitated from the cell lysates using agarose-coupled c-Src antibody, followed by western blotting for anti-phospho-c-Src or anti-c-Src antibody, as a protein loading control. (D) The cells were transiently transfected with c-Src (wild-type; WT) and c-Src (dominant-negative; DN), and stimulated with serotonin (1 μ M) for 10 min. Immunoprecipitation with anti-PLC γ 1 antibody was performed, followed by blotting with phospho-specific antibody which could recognize phosphotyrosine residue of PLC γ 1 (Tyr⁷⁸³), in order to monitor the phosphorylation of endogenous PLC γ 1. The lower panel demonstrates the equivalent loading of total PLC γ 1 in the whole cell lysates utilized in the immunoprecipitation reactions, using anti-PLC γ 1 antibody. The expressions of c-Src were confirmed by blotting for c-Src antibody. (E) The phosphorylation of downstream signaling molecules depends on the activity of PLC. After 24-h starvation, cells were stimulated with serotonin (1 μ M) for 10 min, either in the presence or absence of U73122 (2 μ M). The cell lysates were analyzed via western blotting for anti-phospho-MEK1/2 and ERK1/2 antibodies. Blotting with anti-MEK1/2 and ERK1/2 antibodies was conducted as a protein-loading control. The results were representative of four independent experiments.

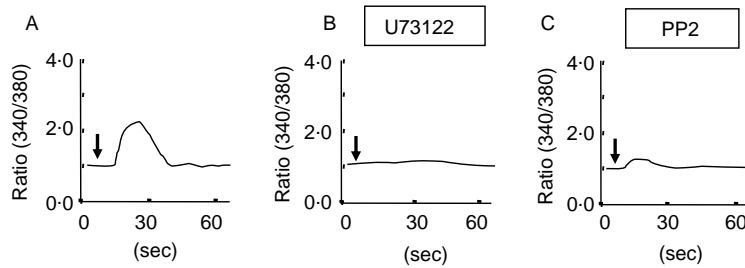


Figure 3 PP2 and U73122 suppress serotonin-induced calcium release. To detect the calcium signal, fura 2-AM (10 μ M) was pre-incubated for 45 min in culture medium. The calcium response was measured after serotonin (1 μ M) treatment in the presence of either U73122 (2 μ M) or PP2 (10 μ M). The arrow indicates the time point at which serotonin treatment was initiated.

stimulation with serotonin (Fig. 3A), and this increase was blocked completely by the administration of U73122 (Fig. 3B). To confirm the involvement of Src kinase in this process, we pretreated the cells with PP2 (10 μ M), and then evaluated the amounts of calcium released. We noted that PP2 pretreatment significantly suppressed the serotonin-induced calcium increase (Fig. 3C). These findings show that both PLC and Src can be associated with serotonin-mediated calcium-related physiological phenomena within the hypothalamus.

PLC γ 1 binds to *c*-Src through the SH223 domain

The primary structures of *c*-Src and PLC γ 1 are provided in Fig. 4A. The linker region between the X and Y domains in PLC γ 1 is extended, and harbors two SH2 domains and one SH3 domain. However, Src kinase is comprised of one SH2, one SH3, and one Tyk domain. Tyrosine phosphorylation is considered a crucial step in the activation of PLC γ 1, and this tyrosine phosphorylation is mediated via the recognition of phosphorylation sites by the SH2 domain. This implies that the protein–protein interactions may be involved in the Src-mediated activation of PLC γ 1. In order to determine whether *c*-Src was associated with PLC γ 1, we performed an immunoprecipitation experiment using the anti-*c*-Src antibody, followed by blotting with the anti-PLC γ 1 antibody. Endogenous PLC γ 1 was first co-immunoprecipitated with *c*-Src upon serotonin stimulation (Fig. 4B). Then, to determine which PLC γ 1 domains were responsible for this binding, we conducted an immunoprecipitation experiment following transient transfection with several forms of PLC γ 1. *c*-Src was co-immunoprecipitated with wild-type PLC γ 1 in a serotonin-dependent manner, but this was not observed with the LIM or SH223 domain-deleted PLC γ 1 mutants (Fig. 4C). These findings indicated that the activation of PLC γ 1 was mediated by direct interaction with *c*-Src via the SH223 domain, and also showed that the enzyme activity of PLC γ 1 was critical with regard to this binding. Focusing on this result, we attempted to determine whether calcium response was affected by transient transfection with several forms of PLC γ 1. Accordingly, serotonin induced a profound

increase in the concentration of intracellular calcium in the cells that had been transfected with wild-type PLC γ 1. However, this increase was dramatically attenuated in the cells transfected with either LIM or SH223 domain-deleted PLC γ 1 mutants (Fig. 4D). Plasmid transfection had no significant effect. The expression of PLC γ 1 was confirmed via western blotting with anti-FLAG antibody (Fig. 4E). These findings support our hypothesis that both the interaction of PLC γ 1 with *c*-Src and the enzyme activity of PLC γ 1 are critical factors in serotonin-mediated calcium release.

PP2 and U73122 suppress the serotonin-mediated release of GnRH

The spontaneous firing of exocytosis vesicles appears to be fundamental to hormone secretion. Its absence in the neurons of 5-hydroxy tryptophan (5-HT) 1B knockout mice (Evrard *et al.* 1999) indicates that serotonin may be involved in this process. In order to determine whether Src-mediated PLC γ 1 activation is involved in hormone secretion, we employed an RIA to characterize the effects of PLC on serotonin-mediated GnRH release. Hypothalamic fragments were rapidly isolated from 10-week-old male mice ($n=6$) and cultured on 96-well tissue culture plates. After equilibration, the tissues were incubated for an additional 1 h in fresh medium containing either vehicle or serotonin (1 μ M). The media were then collected and centrifuged. The amounts of GnRH were increased by nearly fourfold upon stimulation with serotonin. Next, to delineate the signaling pathways involved in the serotonin-mediated release of GnRH, we pretreated the hypothalamic fragments with either PP2 (10 μ M) or U73122 (2 μ M), and then measured the levels of GnRH. The GnRH levels were substantially reduced in the presence of these two inhibitors (Fig. 5A). To determine whether serotonin-mediated GnRH release was correlated with the activation of PLC γ 1, we then attempted to determine if the phosphorylation of PLC γ 1 would be affected by serotonin administration. Although PLC γ 1 was basally phosphorylated in the hypothalamic tissue, its phosphorylation (Tyr⁷⁸³) increased further as a result of stimulation with serotonin (Fig. 5B). The phosphorylation of Src, MEK1/2, and ERK1/2

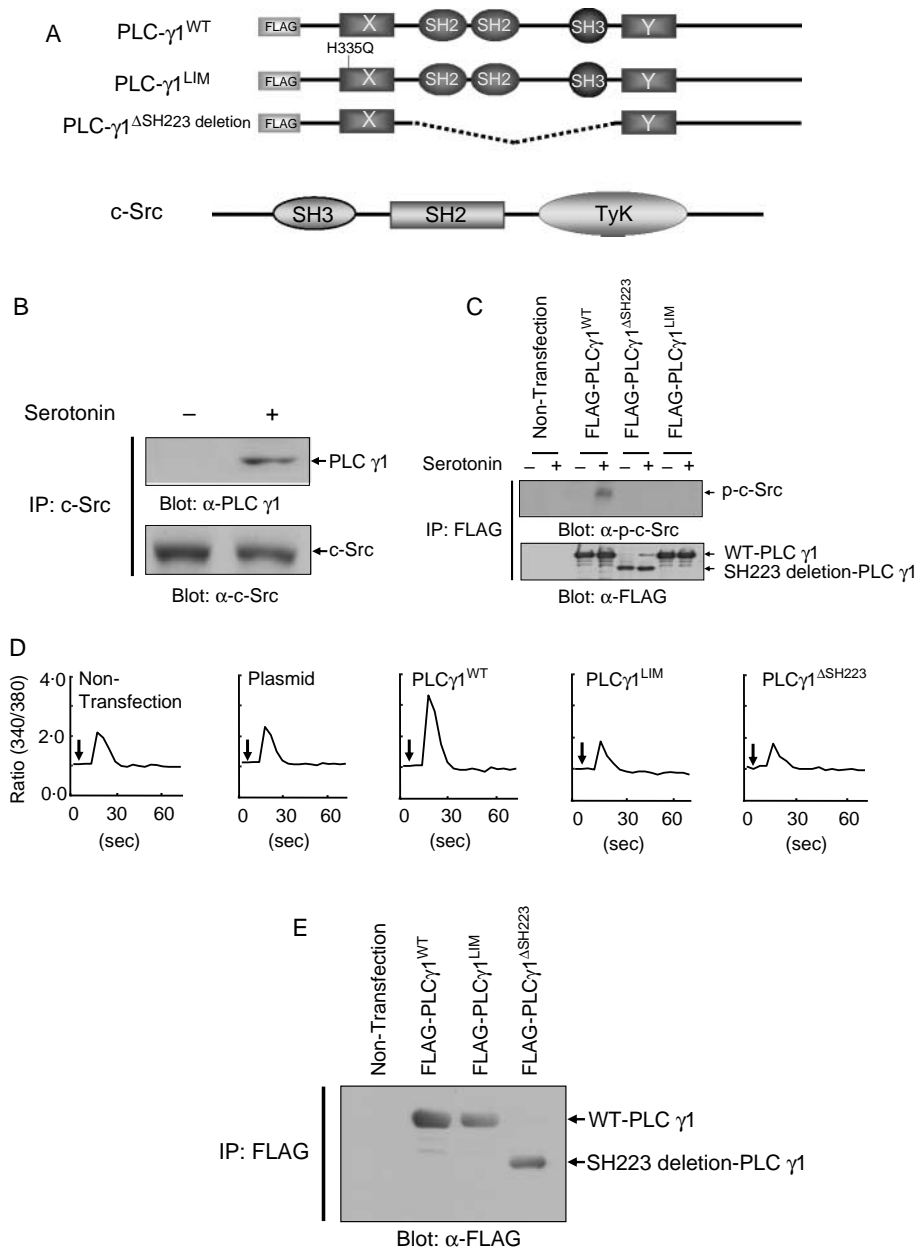


Figure 4 PLC γ 1 binds to c-Src through the SH223 domain. (A) Linear structure of FLAG-tagged wild-type PLC γ 1 (PLC γ 1^{WT}), lipase inactive mutant (PLC γ 1^{LIM}), SH223 domain deletion mutant (PLC γ 1 ^{Δ SH223}), and c-Src. (B) The binding of endogenous c-Src with PLC γ 1. After 24-h starvation, the GT1–7 cells were treated for 10 min with serotonin (1 μ M). c-Src was immunoprecipitated (IP) with anti-c-Src antibody followed by western blotting with anti-PLC γ 1 antibody. The lower panel is the western blotting data, which demonstrates the equivalent loading of total c-Src in the whole cell lysates used in the immunoprecipitation reaction, using anti-c-Src antibody. (C) The binding of PLC γ 1 with phosphorylated c-Src. Cells were transiently transfected with FLAG-wild PLC γ 1, FLAG-LIM PLC γ 1, and FLAG-SH223 domain-deleted PLC γ 1 using LipofectAMINE. After 24-h starvation, the cells were stimulated for 10 min with serotonin (1 μ M). An immunoprecipitation reaction was then conducted using anti-FLAG antibody, and examined via western blotting with anti-phospho-c-Src antibody. (D) Both the SH223 domain and the enzymatic activity of PLC γ 1 are important to serotonin-mediated calcium response. The cells were transiently transfected with plasmid, FLAG-wild PLC γ 1, FLAG-LIM PLC γ 1, and FLAG-SH223 domain-deleted PLC γ 1. After 5 h, the cells were transferred to serum-containing medium, then cultured further for 24 h. The amplitude of calcium concentration upon serotonin (1 μ M) stimulation was determined after 24-h starvation. In order to detect the calcium signal, fura 2-AM (10 μ M) was pre-incubated for 45 min in culture medium. The arrow indicates the time point at which serotonin treatment was initiated. (E) The expressions of PLC γ 1 wild type and mutants. The cells were transiently transfected with FLAG-wild-type PLC γ 1, FLAG-LIM PLC γ 1, and FLAG-SH223 domain-deleted PLC γ 1 mutants. The cell lysates were examined via western blotting for anti-FLAG antibody. The results were representative of four independent experiments.

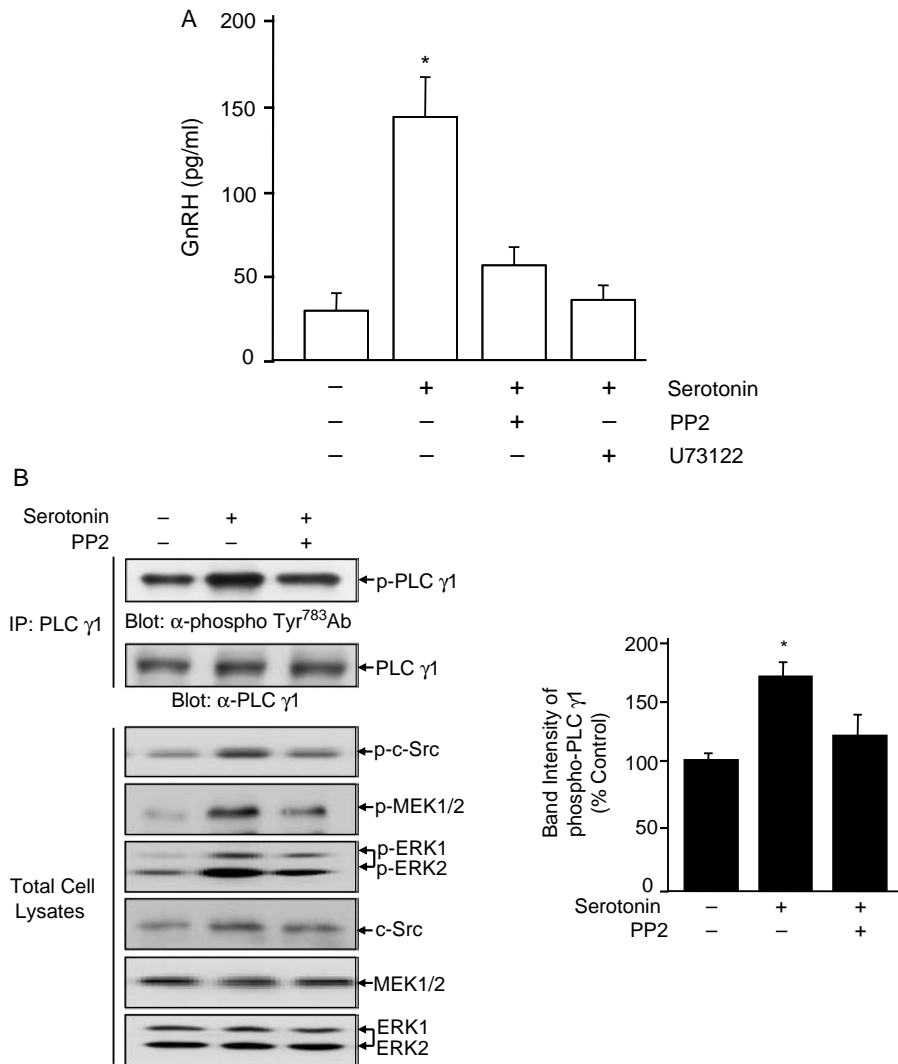


Figure 5 PP2 and U73122 suppress the serotonin-mediated release of GnRH. (A) Serotonin-mediated GnRH release depends on both Src and PLC activity. Hypothalamic fragments were freshly prepared from experimental mice. While these were incubated in DMEM/F12 media, we administered a 30-min pretreatment with either PP2 (10 μ M) or U73122 (2 μ M), followed by 1-h serotonin (1 μ M) treatment. The amounts of secreted soluble GnRH were then determined via the RIA method ($n=6$). GnRH values were expressed as picograms per milliliter. * $P<0.05$, with respect to control (one-way ANOVA with Holm-Sidak comparisons). (B) Activation of PLC γ 1 in the hypothalamic fragments upon serotonin stimulation. Tissue lysates (400 μ g) were prepared after serotonin (1 μ M) stimulation. Immunoprecipitation (IP) with anti-PLC γ 1 antibody was performed, followed by blotting with phospho-specific antibody, which could recognize phosphotyrosine residue of PLC γ 1 (Tyr⁷⁸³), in order to monitor the phosphorylation of endogenous PLC γ 1. The lower panel demonstrates the equivalent loading of total PLC γ 1 in the whole cell lysates utilized in the immunoprecipitation reactions, using anti-PLC γ 1 antibody. Cell lysates (20 μ g) were also blotted with anti-phospho-c-Src, MEK1/2, and ERK1/2 antibodies. Blotting was conducted with anti-c-Src, MEK1/2, and ERK1/2 antibodies as a control for protein loading. Band intensity analysis of PLC γ 1 phosphorylation in the hypothalamic tissue after serotonin treatment ($n=6$). The band intensity signal was quantified using Image Gauge software (version 3.12; Fujifilm). * $P<0.05$, with respect to control (one-way ANOVA with *post hoc* Fisher's test).

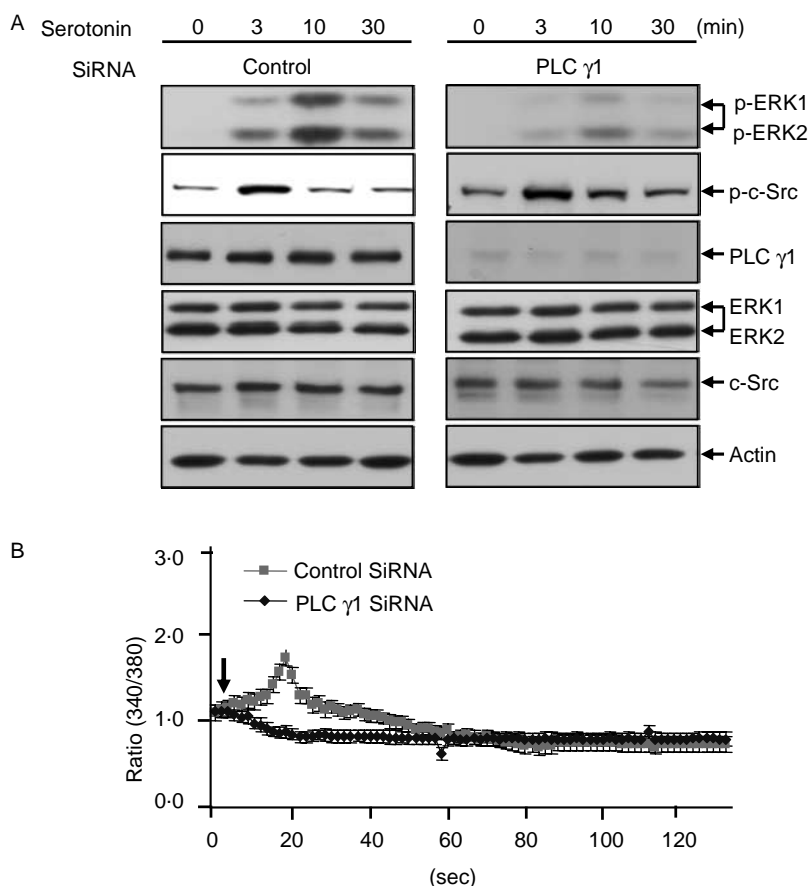


Figure 6 PLC γ 1 knockdown attenuated the serotonin-induced phosphorylation of ERK1/2 and increase in calcium levels. (A) Suppression of ERK1/2 activity in the PLC γ 1 knockdown cells. The cells were transiently transfected with control siRNA or PLC γ 1 siRNA, cultured for an additional 2 days, and then starved for 24 h. The cell lysates were prepared after serotonin (1 μ M) stimulation for the indicated times, and then examined by western blotting for anti-phospho-ERK1/2, c-Src antibodies. Blotting was conducted with anti-c-Src, PLC γ 1, ERK1/2, and actin antibodies as a control for protein loading. The result was representative of four independent experiments. (B) The inhibition of serotonin-mediated calcium responses in the PLC γ 1 knockdown cells. The cells were transiently transfected with control siRNA or PLC γ 1 siRNA, cultured for 2 days, then starved for 24 h. To detect the calcium signal, fura 2-AM (10 μ M) was pre-incubated for 45 min in culture medium. Calcium concentrations were measured after the administration of serotonin (1 μ M). The arrow indicates the time point at which serotonin treatment was initiated.

was also increased in this experimental condition and then suppressed as a result of pretreatment with PP2. Therefore, even though the serotonergic system is just one of several neural inputs that innervate the hypothalamus, our findings indicate that the relation of PLC γ 1 activity with GnRH release in the hypothalamus.

PLC γ 1 knockdown attenuated the serotonin-induced phosphorylation of ERK1/2 and increase in calcium levels

To determine whether PLC γ 1 was, indeed, a prerequisite for the serotonin-mediated signal pathway, we employed RNA

interference techniques. The knockdown of endogenous PLC γ 1 resulted in a noticeable inhibition of serotonin-mediated ERK1/2 phosphorylation (Fig. 6A). Src phosphorylation remained unaffected by PLC γ 1 knockdown, thereby confirming that PLC γ 1 was a downstream event in serotonin-mediated signaling. Specific PLC γ 1 suppression was verified via immunoblotting, whereas actin expression remained unaffected. To confirm this in separate experiments, we compared the calcium response in the PLC γ 1 wild-type cells with that in the PLC γ 1 knockdown cells. The calcium concentration increased by approximately twofold in the wild-type cells, but was negligible in the knockdown cells (Fig. 6B).

These findings, then, provide evidence that endogenous PLC γ 1 is crucial for serotonin-mediated signaling events.

Discussion

The principal finding of this study was that PLC γ 1 is involved in the serotonin-mediated secretion of GnRH. In this study, we determined that the Src-mediated activation of PLC γ 1 and calcium are instrumental in serotonin-mediated GnRH hormone release.

Src kinase is considered to be a common mediator of both G protein-coupled receptors and tyrosine kinase-coupled receptors (Haendeler *et al.* 2003, Stope *et al.* 2004). It has also been reported that Src cascades exploit the Gq-PLC pathway for their activation in the process of calcium-dependent pancreatic exocytosis (Tsunoda *et al.* 2004), thereby indicating that Src operates downstream of PLC γ 1. However, others have asserted that Src is upstream of PLC γ 1 (Bivona *et al.* 2003). Therefore, there remains some controversy regarding the relationship between Src and PLC γ 1. In our study, we have determined that c-Src undergoes tyrosine phosphorylation, and is also associated with PLC γ 1 in the responses of GT1-7 cells to serotonin. Thus, we suggest that c-Src is a novel regulator of PLC γ 1 function, which appears to mediate serotonin-mediated hormone secretion.

The primary assertion of this study is that PLC γ 1 mediates some of the effects of serotonin. The role of PLC has previously been evaluated in conjunction with adrenocorticotrophic hormone, follicle-stimulating hormone, and prolactin (Yajima *et al.* 1997, Rabadan-Diehl & Aguilera 1998). PLCs appear to be responsible for the release of diacylglycerol and inositol triphosphate, and may also contribute to the observed increases in intracellular calcium levels. The contribution of the PLCs to hormone secretion has raised several questions as to which of the characteristics of PLC are relevant to its participation in hormone secretion. Protein-protein interactions and PLC enzyme activity have been considered to be the primary candidates for this crucial attribute. To date, however, the role of the domains in the process of hormone secretion, that of the enzyme activity of PLC γ 1 in this process, have not been elucidated adequately. In the present study, we have established that the serotonin-induced release of intracellular calcium occurred to a less profound degree in the cells transfected with SH223 domain-deleted or LIM PLC γ 1 mutants than in the wild-type PLC γ 1-transfected cells. Additionally, GnRH release was also suppressed by either the Src or the PLC inhibitor. Collectively, our results suggested that both protein-protein interactions and the enzyme activity of PLC γ 1 may play crucial roles in serotonin-mediated GnRH secretion.

Many neurotransmitters, including serotonin, gamma-aminobutyric acid (GABA), and neuropeptide Y, have been shown to modulate GnRH release (Senthilkumar *et al.* 2001). However, it remains unclear as to whether the effects of these neurotransmitters on GnRH secretion are

directly exerted on the GnRH neurosecretory neurons, or indirectly exerted, via multisynaptic pathways. GnRH neurons are assumed to be located in the hypothalamic pre-optic area, and distributed in a scattered fashion. The total number of cells expressing luteinizing hormone-releasing hormone mRNA is approximately 800 in postnatal animals (Hoffman & Finch 1986, Wray *et al.* 1989). These findings indicated that our results might be due to the indirect effects of serotonin on GnRH neurons. Although we were able to demonstrate that PLC γ 1 phosphorylation was increased by exposure to serotonin in the hypothalamic cultures, we were unable to determine precisely whether the data from the hypothalamic cultures could be used to distinguish between direct and indirect serotonin effects on the GT1-7 cells.

The objective of the present study was to ascertain whether or not the secretion of GnRH from hypothalamic tissue was directly regulated by serotonin and, if so, to determine which molecules were actually involved in this process. Our data showed that serotonin evoked GnRH secretion in the hypothalamus in a PLC-dependent manner, which implies that serotonin may directly stimulate the hypothalamus to release GnRH through the PLC pathway, via the modulation of calcium concentrations. We further demonstrated that serotonin-mediated signaling events were attenuated as the result of PLC γ 1 knockdown. Overall, these findings implicated PLC γ 1 in the process of serotonin-mediated GnRH secretion.

In conclusion, we have determined that PLC γ 1 is activated by serotonin in hypothalamic cells, and serotonin-associated GnRH release can be substantially suppressed by inhibitors of either Src or PLC. These results demonstrate that the Src-PLC γ 1 pathway exerts a profound influence on serotonin-mediated GnRH secretion. Future studies should focus on the elucidation of the specificity of the PLC isozymes within the context of serotonin-mediated signaling.

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