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Rapid Effect of Progesterone on the Intracellular Ca^{2+} Oscillation of Immortalized Hypothalamic GT1-7 Cells

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Summary

We herein demonstrate the rapid effect of progesterone (PROG) on the intracellular Ca^{2+} ($[Ca^{2+}]_i$) dynamics in GT1-7 cells. The cells were loaded with Calcium Green-1 AM and single-cell Ca^{2+} imaging was performed by digital microscopic imaging. The frequency of spontaneous Ca^{2+} oscillation was 0.09 Hz. A 20-min incubation with 1 μM PROG significantly suppressed the Ca^{2+} oscillation down to 54% of the control frequency. The inhibitor of the classical PROG receptor, RU-486, completely blocked this PROG-induced suppression of Ca^{2+} oscillation. The Ca^{2+} oscillation was completely blocked by the treatment with nicardipine, an inhibitor of the voltage-sensitive Ca^{2+} channel, but not blocked by the treatment with thapsigargin, an inhibitor of the Ca^{2+} pump of microsomes. Therefore, the PROG-induced suppression of the Ca^{2+} oscillation may be due to modulation of the voltage-dependent Ca^{2+} channel. These results imply that PROG rapidly (within 20 min) drives Ca^{2+} signaling via the nongenomic pathway dependent on classical PROG receptors. The existence of the PROG receptor was confirmed by a Western blot analysis.

Keywords

progesterone, neurosteroid, GT1, Ca^{2+} oscillation, imaging

Introduction

GT1-7 cells are the immortalized hypothalamic neurons that release gonadotropin-releasing hormone (GnRH). Since the regulation of GnRH release by steroid hormones is physiologically important as part of the feedback loop, the effects of steroid hormones on GnRH release have been intensively studied. The repression of GnRH gene expression is caused by progesterone (PROG) (Kepa et al., 1996), estradiol (Roy et al., 1999), testosterone (Shakil et al., 2002), and dexamethasone (Chandran et al., 1996). These effects are mediated by the binding of steroid hormones to the nuclear steroid hormone receptors. In addition to the classical genomic effects of peripheral steroid via intracellular steroid receptors, steroid hormones have been shown to rapidly alter neuronal excitability via a nongenomic pathway by modulating cell surface receptors (Baulieu, 1998; Paul and Purdy, 1992; Wehling, 1997). In addition, 17β-estradiol has been reported to act on GT1-7 cells in acute manners (Morales et al., 2003).

PROG exemplifies the types of steroid hormones that act through non-genomic means both on neurons and on other type of cells. In neurons, PROG enhances 5-HT1A receptor agonists-induced GnRH release by acting on the plasma membrane in fetal rat hypothalamic cells (Hery et al., 1995), blocks ionotropic receptor responses in the ganglion cells of Aplysia (Takashima et al., 2002), and rapidly suppresses GnRH and luteinizing hormone secretion mediated by the classical PROG receptor in ewes (Skinner et al., 1998). In other types of cells, PROG inhibits oxytocin receptor function by directly binding to the oxytocin receptor (Grazzini et al., 1998), stimulates protein tyrosine phosphorylation by the PROG receptor on the cell surface of human sperm (Tesarik et al., 1993), and induces the maturation of *Xenopus* oocytes by inducing the association of the classical PROG receptor with phosphatidylinositol 3-kinase (Bagowski et al., 2001).

Since the release of GnRH requires an elevation of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) following membrane depolarization, changes in $[Ca^{2+}]_i$ have been examined as an indicator of GnRH release (Kaneishi et al., 2002; Kawahara et al., 2000; Uemura et al., 1997). The amplitude of the intra-

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cellular Ca^{2+} oscillation in the GT1-7 cells was increased at 72 h after the 100 nM PROG application (Sekiguchi et al., 2003). We herein report a novel rapid (< 20-min) effect of PROG on the frequency of $[Ca^{2+}]_i$ oscillation in GT1-7 cells via classical PROG receptors.

Materials and methods

Chemicals

Cremophor EL, polyethylenimine, PROG and RU-486 were purchased from Sigma (USA). Nicardipine and thapsigargin were purchased from Wako Pure Chemicals (Japan). DMEM, F12 and fetal calf serum were purchased from Gibco (Invitrogen, USA). Calcium Green-1/AM was purchased from Molecular Probes (USA). Polyclonal antibodies against PROG receptors were purchased from DAKO (USA). Fat-free skim milk (Block Ace) was purchased from Dainippon Pharmaceutical (Japan).

Cell cultures

GT1-7 immortalized hypothalamic neurons (provided by Prof. Yoshihiro Ohta, Tokyo University of Agriculture and Technology at Tokyo) were grown in DMEM/F12 supplemented with 10% fetal bovine serum. After enzymatic dissociation, cells were plated on glass coverslips coated with polyethylenimine at a density of 4×10^4 cells/cm^2. Prior to performing the intracellular free Ca^{2+} measurements, the cells were cultured and differentiated in serum-free Dulbecco's modified Eagle's medium in the absence of phenol red for 4 – 5 days.

Ca^{2+} measurements

The intracellular Ca^{2+} dynamics in the GT1-7 cells were measured according to the method described previously (Sekiguchi et al., 2003) with a slight modification. The cultures were loaded with 3 μM Calcium Green-1/AM for 30 min at 37℃ in the presence of 0.03% cremophor EL in a balanced salt solution (BSS: 130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 0.01 mM HEPES, 5.5 mM glucose and 10 μM glycine). After dye loading, the cells were rinsed 3 times with BSS, and intracellular Ca^{2+} ($[Ca^{2+}]_i$) was monitored by single-cell fluorescence imaging with a digital fluorescence microscope system consisting of an inverted microscope (TMD-300, Nikon, Japan) and a CCD camera (C2400-77, Hamamatsu Photonics, Japan). The cells were excited with a 470 – 490 nm light with a xenon lamp and optical filter, and fluorescence above 520 nm was measured. A series of image frames were acquired at an interval of 3 s under computer control. Except for the exposure time (0.1 s), we blocked the illumination with a mechanical shutter to avoid any potential cellular damage.

An analysis of fluorescence images was performed with the Argus-50/CA system (Hamamatsu Photonics). The acquired images were stored in a hard disk with a spatial resolution of 256 × 256 pixels with 8-bit depth. For generation of the time-dependent curves, the fluorescence intensity data in each area (20 × 20 pixels) were averaged with a 3-s resolution. The changes in $[Ca^{2+}]_i$ were expressed as the normalized fluorescence intensity $F(t)/F_{min}$. $F(t)$ and F_{min} represent the fluorescence intensity of Calcium Green-1 at time t, and the minimum fluorescence intensity during t = -3 – 0 min, respectively. All observations were performed at 37℃. In order to analyze the rapid effect of PROG, we compared the frequency of Ca^{2+} oscillation over 3 min, before (Freq(-3 – 0 min)) and 20 min after the administration of PROG (Freq(20 – 23 min)).

Western Immunoblot

GT1-7 cells were homogenized in 0.32 M sucrose in HEPES buffer (pH 7.3) with 25 mM KCl, 4 mM EDTA, 50 mM NaF, 1 mM sodium orthovanadate, protease inhibitors PMSF (0.5 mM) and leupeptin (0.1 mM) and centrifuged at 830×g for 10 min at 4℃. The supernatant was subjected to centrifugation at 10,000×g for 20 min at 4℃. These fractions were suspended in 125 mM Tris-HCl buffer (pH 6.8), containing 5 mM 2-mercaptoethanol, 10% sucrose, 6% sodium dodecylsulfate and 0.002% bromophenol blue. The fractions were subjected to electrophoresis using a 10% polyacrylamide gel. After transfer to polyvinylidene fluoride membranes (Immobilon-P; Millipore Co., USA), the blots were probed with antisera against PROG receptor (1/1600, DAKO, DK) for 12 – 18 h at 4℃, and incubated with horseradish peroxidase conjugated with goat anti-rabbit IgG (Cell Signaling, USA). The membranes were treated with ECL plus Western blotting detection reagents (Amersham, USA), and chemiluminescence photographs were taken with the LAS-3000 Image analyzer (Fujifilm, Japan).

Statistical Analysis

The data are expressed as the mean ± SEM. A comparison of intracellular Ca^{2+} oscillation between the control and treatment groups was performed with

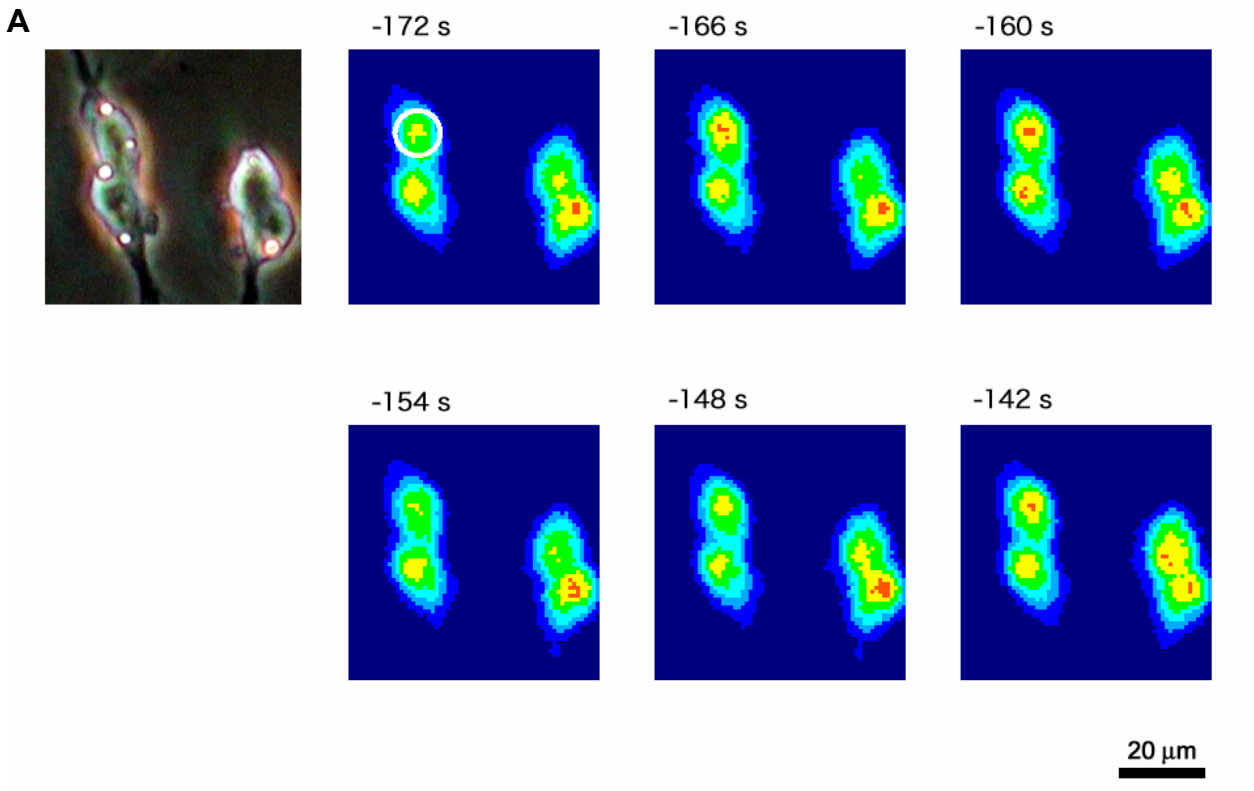


Fig. 1. Time-dependent images and time course of spontaneous oscillation of intracellular Ca^{2+} in GT1-7 cells. (A) Representative pseudocolor Ca^{2+} images of GT1-7 cells loaded with Calcium Green-1. Scale bar, 20 μm. Typical time-course profile of $F(t)/F_{min}$ in GT1-7 cells (B) before and (C) 20 min after the administration with 100 nM PROG (indicated by circle in Figure 1A). $F(t)/F_{min}$ is the normalized fluorescence intensity. $F(t)$ and F_{min} represent the fluorescence intensity of Calcium Green-1 at time t, and the minimum fluorescence intensity during t = -3 – 0 min. Arrow-heads (B and C) indicate Ca^{2+} spikes.

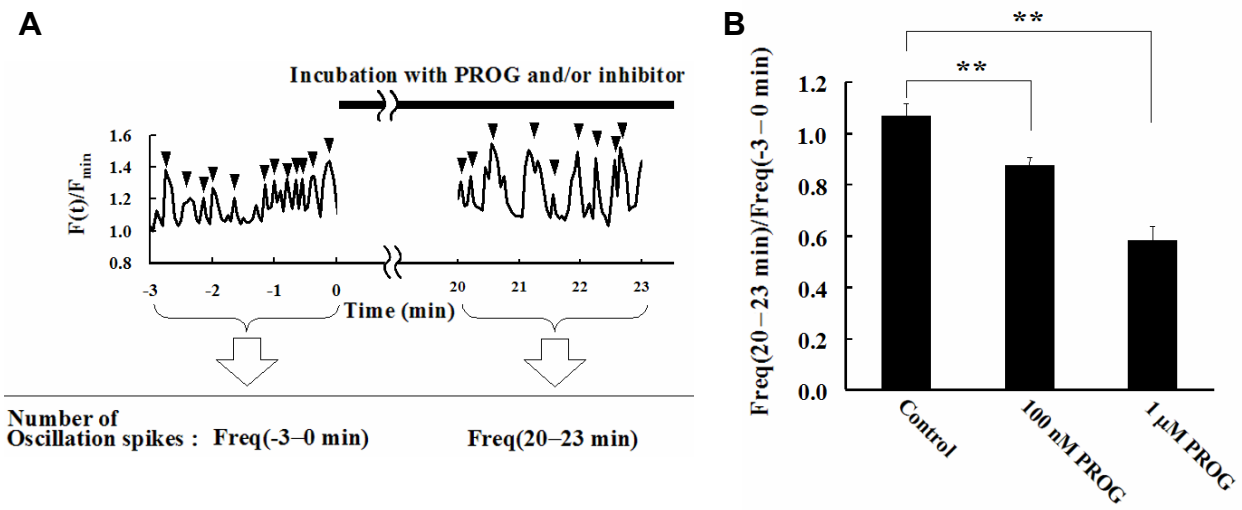
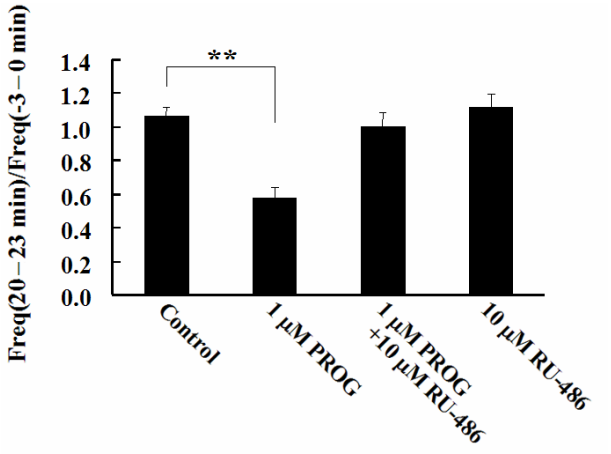


Fig. 2. Effect of PROG treatments on the spontaneous oscillation of intracellular Ca^{2+} in GT1-7 cells. Figure 2A shows the experimental design for the measurement of PROG effects on Ca^{2+} oscillation. $F(t)/F_{min}$ is the normalized fluorescence intensity. F(t) and F_{min} represent the fluorescence intensity of Calcium Green-1 at time t, and the minimum fluorescence intensity during t = -3 – 0 min. (B) PROG effects on the ratio of the frequency of Ca^{2+} oscillation, Freq(20 – 23 min)/ Freq(-3 – 0 min). GT1-7 cells were treated for 20 min without PROG (control, *N* = 57), with 100 nM PROG (*N* = 59), and with 1 μM PROG (*N* = 30). *N* is the number of cells analyzed. The results are reported to be the mean ± SEM. The significance of the drug effects was confirmed using ANOVAs (**p < 0.01).

Fig. 3. Effect of PROG on the ratio of the frequency of Ca^{2+} oscillation, Freq (20 – 23 min)/Freq(-3 – 0 min), in GT1-7 cells. GT1-7 cells were treated for 20 min without PROG (control, *N* = 59), and with 1 μM PROG (*N* = 30), with 1 μM PROG and 10 μM RU-486 (*N* = 20), and with 10 μM RU-486 (*N* = 23). *N* is the number of cells analyzed. Results are reported as the mean ± SEM. The significance of the drug effects was confirmed by using ANOVAs (**p < 0.01).



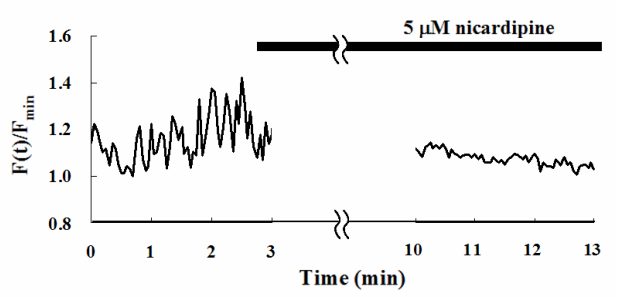


Fig.4. Effect of Ca^{2+} channel blocker on Ca^{2+} oscillation in GT1-7 cells. Typical time course profiles of Ca^{2+} oscillation in GT1-7 (A) before and (B) 10 min after the administration with 5 μM nicardipine, which is a block of the voltage-dependent calcium channels. $F(t)/F_{min}$ is the same as that in Fig. 2.

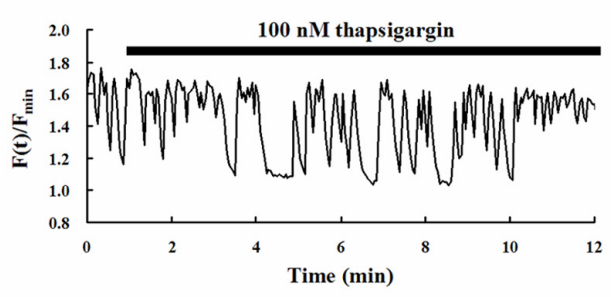


Fig. 5. Effect of depletion of intracellular Ca^{2+} stores by 100 nM thapsigargin, the inhibitor of Ca^{2+} pump in the endoplasmic reticulum, in GT1-7 cells. The horizontal black bar indicates treatment with 100 nM thapsigargin, an inhibitor of the Ca^{2+} pump in the endoplasmic reticulum. $F(t)/F_{min}$ is the same as that in Fig. 2.

One-way analysis of variance (ANOVA) accompanied by post-hoc Tukey-Kramer's test. Differences were considered significant given a P value of < 0.01.

Results

Rapid effects of PROG on intracellular Ca^{2+} oscillation

We observed the spontaneous Ca^{2+} oscillation in GT1-7 cells, as shown in Fig. 1A and 1B, using single-cell imaging. For the 55 – 70% of cells that showed Ca^{2+} oscillation, the frequency of Ca^{2+} oscillation was 0.092 ± 0.007 Hz. The half-width of each Ca^{2+} spike was around 5 – 10 s.

In order to analyze the rapid effect of PROG as shown in Fig. 1C, we compared the frequency of Ca^{2+} oscillation over 3 min, before (Freq(-3 – 0 min)) and 20 min after the administration of PROG (Freq (20 – 23 min)) in GT1-7 cells (Fig. 2A, 2B). PROG significantly decreased the ratio of oscillation frequency, Freq(20 – 23 min)/Freq(-3 – 0 min), from 1.067 ± 0.047 (control) to 0.872 ± 0.033 (100 nM PROG) and 0.581 ± 0.059 (1 μM PROG) within 20 min (Fig. 2B).

Effect of PROG receptor blockage

RU-486 (classical PROG receptor antagonist) completely blocked the attenuation effect of PROG on the frequency of the Ca^{2+} oscillation (Fig. 3). RU-486 alone had no effect on the frequency of the Ca^{2+} oscillation.

Effect of voltage-dependent Ca^{2+} channel blockage on Ca^{2+} oscillation

In order to investigate the mechanism of Ca^{2+} oscillation, the source of Ca^{2+} was investigated. The Ca^{2+} oscillation in the GT1-7 cells was completely blocked by 5 μM nicardipine, a blocker of the L-type voltage-dependent Ca^{2+} channel, within 5 min (Fig. 4). In addition, the Ca^{2+} oscillation was blocked when Ca^{2+} in the extracellular medium was eliminated or chelated by EGTA (data not shown). On the other hand, the treatment of thapsigargin (100 nM), an inhibitor of Ca^{2+} pump in the endoplasmic reticulum, did not affect the Ca^{2+} oscillation (Fig. 5).

Western immunoblot analysis of PROG receptor

The presence of the PROG receptor was demonstrated as a single band in the GT1-7 cells using a Western immunoblot analysis (Fig. 6). The electrophoretic mobility of the PROG receptor band indicated a molecular mass of approximately 100 kDa in both the differentiated and the non-differentiated cells. Almost no differences in the expression level for PROG receptor were observed between the differentiated and the non-differentiated cells.

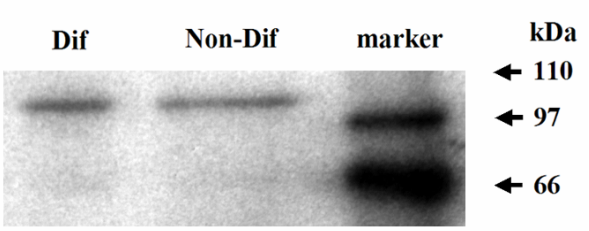


Fig. 6. A Western immunoblot analysis of the PROG receptor in GT1-7 cells. From left to right: PROG receptor in differentiated GT1-7 cells (Dif), non-differentiated GT1-7 cells (Non-Dif), and molecular marker proteins. On each lane, 10 μg protein was applied.

Discussion

The current study demonstrated that the activation of the classical PROG receptor by PROG induced the rapid suppression of the frequency of Ca^{2+} oscillation in the GT1-7 cells, by means of single-cell $[Ca^{2+}]_i$ imaging. The rapid effect by 0.1 and 1 μM PROG on intracellular Ca^{2+} oscillation was observed within only 20 min.

Such a rapid modulation of Ca^{2+} oscillation by PROG is a novel observation and requires the rapid signaling pathway driven by PROG receptors. Expression of the classical PROG receptor in the GT1-7 cells was indicated by a Western blot analysis (Fig. 6). Involvement of the classical PROG receptor in the attenuation on the frequency of Ca^{2+} oscillation was indicated by the inhibition of the attenuation with RU-486 (inhibitor of classical PROG receptor) (Fig. 3). This rapid signaling pathway is probably different from well-known steroid receptor actions that involve migration of the PROG receptors to the nucleus, subsequent binding to DNA and gene transcription (genomic pathways). Genomic mechanisms of PROG action are generally characterized by their slow time course, usually taking several hours or even days (Wiebe et al., 1997).

PROG inhibits GnRH pulse frequency within 1 h in estradiol-primed ewes (Skinner et al., 1998).

This action of PROG appears to be mediated by the classical PROG receptor, because the PROG effects are blocked by RU-486 (Skinner et al., 1998). Note that the blockage of Ca^{2+} oscillation inhibits episodic GnRH release from GT1-7 neurons, indicating that Ca^{2+} oscillation is essential for GnRH release (Martinez de la Escalera et al., 1992). However, the proteins, such as kinases or G proteins, possibly involved in these rapid signaling cascades have not yet been well analyzed.

The area downstream of the PROG receptor in the rapid signal cascade is not yet well known. It is also unclear whether or not the classical PROG receptor is located in the plasma membrane of GT1-7 cells. A portion of the PROG receptor molecules may act rapidly around the plasma membrane without moving to the nuclei (Haseroth et al., 2000). For example, in the midbrain ventral tegmental area, the facilitation of the lordosis response by PROG conjugated to BSA has been demonstrated within 10 min (Frye and Vongher, 1999). The rapid signaling pathway may be a nongenomic one that involves some kinases. Protein kinase A (PKA) might be involved in the rapid action of PROG. Although the exposure of the rat heart to 17β-estradiol rapidly (within 10 min) increases the PKA-dependent Ca^{2+} influx and induces the activation of voltage-dependent Ca^{2+} channels in rat heart cells, PROG application does not activate PKA (Buitrago et al., 2000). Erk MAP kinase may be another candidate for rapid PROG signaling, because the rapid estradiol action is mediated by Erk MAP kinase in estradiol-induced synaptogenesis (Mukai et al., 2007). Further investigations are necessary in GT1 cells to identify the kinases that may be involved in rapid PROG signaling.

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