

Critical Role of Phospholipase C γ 1 in the Generation of H₂O₂-evoked [Ca²⁺]_i Oscillations in Cultured Rat Cortical Astrocytes*^[5]

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Reactive oxygen species, such as the superoxide anion, H₂O₂, and the hydroxyl radical, have been considered as cytotoxic by-products of cellular metabolism. However, recent studies have provided evidence that H₂O₂ serves as a signaling molecule modulating various physiological functions. Here we investigated the effect of H₂O₂ on the regulation of intracellular Ca²⁺ signaling in rat cortical astrocytes. H₂O₂ triggered the generation of oscillations of intracellular Ca²⁺ concentration ([Ca²⁺]_i) in a concentration-dependent manner over the range 10–100 μM. The H₂O₂-induced [Ca²⁺]_i oscillations persisted in the absence of extracellular Ca²⁺ and were prevented by depletion of intracellular Ca²⁺ stores with thapsigargin. The H₂O₂-induced [Ca²⁺]_i oscillations were not inhibited by pretreatment with ryanodine but were prevented by 2-aminoethoxydiphenyl borate and caffeine, known antagonists of inositol 1,4,5-trisphosphate receptors. H₂O₂ activated phospholipase C (PLC) γ 1 in a dose-dependent manner, and U73122, an inhibitor of PLC, completely abolished the H₂O₂-induced [Ca²⁺]_i oscillations. In addition, RNA interference against PLC γ 1 and the expression of the inositol 1,4,5-trisphosphate-sequestering “sponge” prevented the generation of [Ca²⁺]_i oscillations. H₂O₂-induced [Ca²⁺]_i oscillations and PLC γ 1 phosphorylation were inhibited by pretreatment with dithiothreitol, a sulfhydryl-reducing agent. Finally, epidermal growth factor induced H₂O₂ production, PLC γ 1 activation, and [Ca²⁺]_i increases, which were attenuated by *N*-acetylcysteine and diphenylethylidonium and by the overexpression of peroxiredoxin type II. Therefore, we conclude that low concentrations of exogenously applied H₂O₂ generate [Ca²⁺]_i oscillations by activating PLC γ 1 through sulfhydryl oxidation-dependent mechanisms. Furthermore, we show that this mechanism underlies the modulatory effect of endogenously produced H₂O₂ on epidermal growth factor-induced Ca²⁺ signaling in rat cortical astrocytes.

H₂O₂ is a member of the reactive oxygen species (ROS),⁶ which cause oxidative damage to cellular components such as lipids, nucleic acids, and proteins. Therefore, H₂O₂ has generally been considered to be cytotoxic and hazardous to living organisms. However, a growing body of evidence suggests that H₂O₂ serves as an intracellular signaling molecule modulating various physiological functions (1). Cells possess mechanisms that can rapidly synthesize and destroy H₂O₂ in response to receptor stimulation. For example, stimulation of membrane receptors of various growth factors, such as transforming growth factor- β 1, platelet-derived growth factor, and epidermal growth factor (EGF) triggers the rapid and transient production of H₂O₂ (2–5). H₂O₂ generated in response to receptor stimulation has been shown to play an important role in regulating various normal cell functions, such as cell proliferation, platelet aggregation, and vasodilation (6–8). In addition to this, exogenous addition of H₂O₂ at low concentrations affects the functions of various ion channels and other proteins involved in signal transduction (8–10). Therefore, H₂O₂ fulfills the prerequisites for being considered as a genuine intracellular messenger.

Recently, a great deal of attention has focused on the sensitivity of the mechanisms responsible for Ca²⁺ mobilization in response to changes in the cellular redox state. Ca²⁺ plays a pivotal role in the regulation of a diverse range of cellular functions, such as muscle contraction, secretion, synaptic plasticity, cell proliferation, and cell death (11). Many hormones and neurotransmitters increase intracellular Ca²⁺ concentration ([Ca²⁺]_i) by mobilizing Ca²⁺ from intracellular stores and by inducing an influx from the extracellular space (12, 13). H₂O₂ has been shown to enhance the activity of L-type Ca²⁺ channels (10). Peroxide can also stimulate the mobilization of Ca²⁺ in many cell types by modifying Ca²⁺ release channels, such as TRPM2 (14), ryanodine receptors (15), and inositol 1,4,5-trisphosphate (IP₃)-dependent Ca²⁺ channels (16). In addition, H₂O₂ can modify the activity of Ca²⁺ pumps involved in Ca²⁺ homeostasis, such as the sarcoendoplasmic reticulum Ca²⁺-ATPase (SERCA) (17) and plasma membrane Ca²⁺-ATPase (17). Furthermore, enzymes involved in Ca²⁺ signaling pathways, such as phospholipase C (PLC) γ 1 (18) and phospholipase D (19) are also targets. However, most of the previous studies employed high concentrations of H₂O₂, and it is questionable whether such diverse actions of H₂O₂ on

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^[5] The on-line version of this article (available at <http://www.jbc.org/>) contains supplemental Fig. S1.

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⁶ The abbreviations used are: ROS, reactive oxygen species; PLC γ 1, phospholipase C γ 1; [Ca²⁺]_i, intracellular Ca²⁺ concentration; IP₃, inositol 1,4,5-trisphosphate; EGF, epidermal growth factor; SERCA, sarcoendoplasmic reticulum Ca²⁺ ATPase; PMCA, plasma membrane Ca²⁺ ATPase; PLC β 1, phospholipase C β 1; DTT, dithiothreitol; DPI, diphenylethylidonium; NAC, *N*-acetylcysteine; DCF, 2',7'-dichlorofluorescein diacetate; Prx II, peroxiredoxin type II; GFP, green fluorescent protein; siRNA, small interfering RNA; PSS, physiological salt solution; 2-APB, 2-aminoethoxydiphenyl borate; MEM, minimum essential medium; RNAi, RNA interference.

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calcium signaling also occur under normal physiological conditions. Therefore, it will be of great value to identify the target molecules modulated by physiologically relevant concentrations of H₂O₂.

Astrocytes, the major glial cell type in the mammalian brain, participate in a variety of important functions in the central nervous system. As in virtually all other cell types, astrocytes also use Ca²⁺ signaling to mediate a large spectrum of physiological responses. Elevation of [Ca²⁺]_i in response to stimulation of various receptors causes the release of neurotransmitters, such as glutamate and ATP, and plays an important role in the exchange of information with neurons and the regulation of local blood flow (20, 21). In contrast to Ca²⁺ signaling, much less attention has been given to redox signaling in astrocytes. Because ROS are involved in the pathogenesis of neurodegenerative diseases and astrocytes have been shown to possess high antioxidant activities, many studies have focused on the protective roles of astrocytes against oxidative stress-induced neuronal cell death (22–24). However, despite the lack of information about the physiological roles of ROS in astrocytes, NADPH oxidase was shown to be involved in the generation of H₂O₂ and cell survival in this cell type (25). Given the widespread involvement of H₂O₂ in modulating Ca²⁺ signaling cascades, it is tempting to speculate that astrocytes may also use redox signaling to modify their Ca²⁺ signaling.

Therefore, in the present study, we sought to investigate the roles of H₂O₂ in Ca²⁺ signaling in cultured rat astrocytes. Our results indicate that a low, physiologically relevant concentration of H₂O₂ (30 μ M) induces [Ca²⁺]_i oscillations in a PLC γ 1- and IP₃-dependent manner. In addition, H₂O₂ produced endogenously by EGF receptor stimulation is involved in the modulation of Ca²⁺ signaling in rat astrocytes.

EXPERIMENTAL PROCEDURES

Materials—H₂O₂, dithiothreitol (DTT), thapsigargin, 2-aminooxydiphenyl borate (2-APB), ryanodine, *N*-acetylcysteine (NAC), diphenylethylideneiodonium (DPI), caffeine, histamine, EGF, U73122, U73343, and 2',7'-dichlorofluorescein diacetate (DCF) were purchased from Sigma. Minimum essential medium (MEM) containing 100 mg/liter sodium succinate and 75 mg/liter succinic acid, trypsin-EDTA, Opti-MEM, penicillin, streptomycin, and fetal bovine serum were purchased from Invitrogen. Fura-2-acetoxymethyl ester was purchased from Teflabs (Austin, TX). All other chemicals were of reagent grade. The polyclonal antibody against PLC γ 1 and monoclonal antibody against phosphotyrosine (PY783) of PLC γ 1 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-PLC β 1 polyclonal antibody was kindly provided by Dr. Shmuel Muallem (University of Texas Southwestern Medical Center, Dallas).

Cell Cultures—Primary cultures of cortical astrocytes were prepared from neonatal (12–24 h) Wistar rats. Briefly, cortices were dissected, and the tissues were minced and mechanically dissociated. Then the isolated cells were plated on 60-mm culture dishes and maintained at 37 °C in a humidified 5% CO₂ and 95% air for 2–3 weeks. For [Ca²⁺]_i measurements, cells were cultured on 0.01% poly-L-lysine-coated cover glasses in 60-mm dishes for 7–10 days. The culture medium consisted of MEM supplemented with 2 mM glutamine, 25 mM glucose, 100 μ g/ml penicillin, 25 ng/ml streptomycin, and 10% fetal bovine serum. The culture medium was replaced every 3 days. Cells were serum-starved for 2 days before each experiment.

Expression of IP₃ Sponge and Peroxiredoxin Type II (Prx II)—Astrocytes were transiently transfected with a green fluorescent protein (GFP)-tagged high affinity (R441Q) or low affinity (K508A) IP₃-sequestering sponge (26), or were cotransfected with Prx II (1 μ g/ml; kindly provided by Professor S. W. Kang, Ewha Womans University, Seoul, Korea) and eGFP-N1 (1.2 μ g/ml; Clontech) using Lipofectamine 2000

reagent (Invitrogen), according to the manufacturer's instructions. Cells were incubated for 48 h at 37 °C, in a 5% CO₂ atmosphere with saturated humidity to allow expression of the construct before the experiment. The expression of each protein was confirmed by GFP fluorescence.

Transfections of siRNA-PLC γ 1—Construction of a small interfering RNA (siRNA) for PLC γ 1 (siRNA-PLC γ 1) was described before (27). The pSUPER vector for siRNA was purchased from OligoEngine (Seattle, WA). Cells were cotransfected with siRNA-PLC γ 1 (1 μ g/ml) and eGFP-N1 (1.2 μ g/ml) using Lipofectamine 2000 reagent and cultured for 48 h in serum-free MEM. Depletion of endogenous PLC γ 1 by siRNA was confirmed by immunoblot or GFP fluorescence.

Western Blot Analysis—Astrocytes transfected with or without siRNA-PLC γ 1 and eGFP-N1 were stimulated with H₂O₂ or EGF for the indicated times in the physiological salt solutions (PSSs) containing 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM HEPES, and 10 mM glucose (pH 7.4). Cells were then lysed at 4 °C in lysis buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris, 10 mM NaF, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml aprotinin, 1 mM leupeptin, and 1 mM sodium orthovanadate) and centrifuged at 12,000 rpm for 10 min at 4 °C. The samples were subjected to SDS-PAGE and subsequently transferred to nitrocellulose membranes. The membranes were incubated with specific antibodies against PLC γ 1, phosphospecific tyrosine 783, PLC β 1, and actin, and the proteins were detected by ECL (Amersham Biosciences). The intensity of bands was quantified using MetaMorph Analysis System (Universal Imaging Co., Downingtown, PA).

[Ca²⁺]_i Measurements—For [Ca²⁺]_i measurements, attached cells were loaded with fura-2 by incubation with 3.5 μ M fura-2-acetoxymethyl ester in PSS equilibrated with 100% O₂ for 40 min at room temperature. The cells were then washed twice and rested for at least 20 min before use. The fura-2-loaded cells were mounted on the stage of an inverted microscope (Nikon, Tokyo, Japan) for imaging. The cells were superfused at a constant perfusion rate with the PSS. In Ca²⁺-free solutions, CaCl₂ was omitted, and 1 mM EGTA was added. The excitation wavelength was alternated between 340 and 380 nm, and the emission fluorescence was monitored at 510 nm with a CCD camera using MetaFluor system (Universal Imaging Co., Downingtown, PA). Fluorescence images were obtained at 4-s intervals. Background fluorescence was subtracted from the raw signals at each excitation wavelength, and the values of [Ca²⁺]_i were calculated using the equation described previously (28).

ROS Imaging—ROS levels were measured using the fluorescence probe DCF. In brief, cells were incubated for 5 min in the presence of 5 μ g/ml DCF and washed in Hanks' balanced salt solution. DCF fluorescence was measured using a confocal laser-scanning microscope (Leica, Buffalo, NY) with an excitation wavelength at 488 nm and an emission at 525 nm. To avoid photo-oxidation of DCF, the fluorescence images were collected using a single rapid scan, and identical settings were used for all samples.

Data Analysis—The results are presented as mean \pm S.E. Statistical analysis was performed by unpaired Student's *t* test. *p* values lower than 0.05 were considered to be statistically significant.

RESULTS

H₂O₂ Mobilizes Ca²⁺ in Cultured Rat Cortical Astrocytes in a Concentration-dependent Manner—The effect of H₂O₂ on Ca²⁺ mobilization was examined in fura-2-loaded cultured rat astrocytes. Exposure of the cells to H₂O₂ at concentrations lower than 3 μ M failed to increase [Ca²⁺]_i at least for 20 min (Fig. 1A). However, 10 μ M H₂O₂ was shown to induce [Ca²⁺]_i oscillations in 34.7 \pm 8.4% of the 18 tested cells, and the

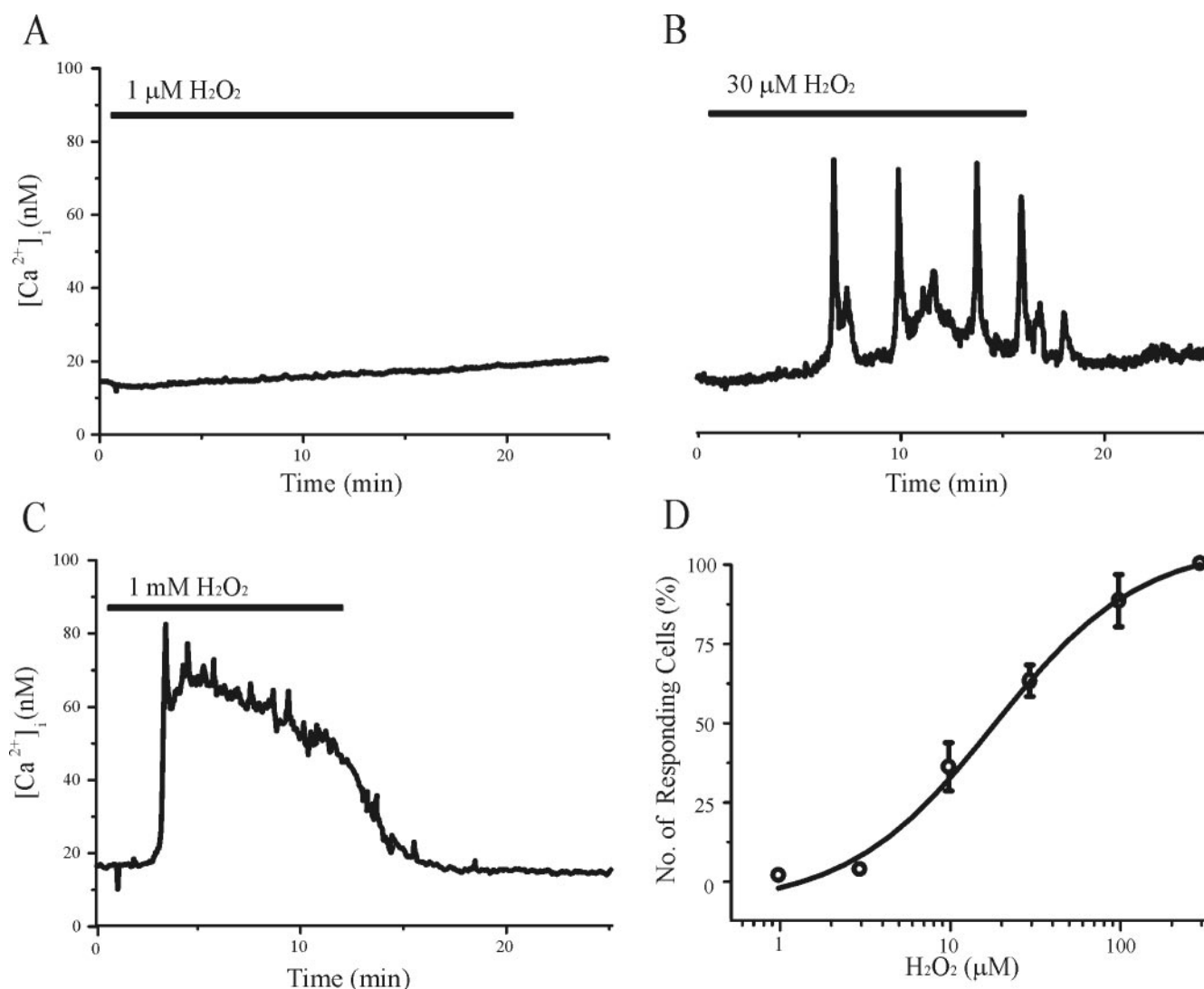


FIGURE 1. H₂O₂ mobilizes Ca²⁺ in a dose-dependent manner in cultured rat cortical astrocytes. Cells were loaded with fura-2 as described under "Experimental Procedures," and changes in [Ca²⁺]_i were measured using ratiometric fluorescence imaging. Cells were exposed to 1 μM (A), 30 μM (B), or 1 mM (C) H₂O₂. For each panel, the trace is representative of 18–20 cells in four or five independent experiments. D, the number of cells responding to H₂O₂ is plotted as a function of the added H₂O₂ concentrations (1, 3, 10, 30, 100, and 300 μM). Data are expressed as the percentage of responding cells. Results are depicted as mean ± S.E.

number of the responding cells increased concentration-dependently (60.9 ± 5.8% for 30 μM, 84.9 ± 10.2% for 100 μM, and 93.1 ± 1.7% for higher than 300 μM H₂O₂; Fig. 1D, *n* = 18–20).

In general, as shown in Fig. 1, B and C, concentrations of H₂O₂ less than 30 μM induced oscillations of [Ca²⁺]_i, whereas doses higher than 300 μM caused spike and plateau type [Ca²⁺]_i increases. However, in some cases, 30 μM H₂O₂ also caused spike and plateau signals. The average lag time between exposure to 10 μM H₂O₂ and the generation of Ca²⁺ responses was 7.4 ± 1.0 min, and it tended to decrease as the concentration of H₂O₂ was increased (5.1 ± 0.5 min for 30 μM, 4.0 ± 0.4 min for 100 μM, and 3.2 ± 0.4 min for 300 μM). Because 30 μM H₂O₂ did not induce cell death (data not shown) and generally produced reliable and reversible [Ca²⁺]_i oscillations, we chose this concentration to analyze the mechanism by which peroxide-stimulated Ca²⁺ signaling occurred in subsequent experiments.

Thapsigargin-releasable, IP₃-sensitive Ca²⁺ Stores Are Responsible for H₂O₂-induced [Ca²⁺]_i Oscillations—To identify the source of the Ca²⁺ mobilization, Ca²⁺ was removed from the bath solution and then 30 μM H₂O₂ was added. As shown in Fig. 2A, [Ca²⁺]_i oscillations persisted in the absence of extracellular Ca²⁺ (*n* = 18), suggesting that intracellular

Ca²⁺ stores were the main source for H₂O₂-induced [Ca²⁺]_i oscillations. Depletion of the intracellular Ca²⁺ stores with thapsigargin, a specific inhibitor of SERCA, prevented H₂O₂-induced [Ca²⁺]_i oscillations (*n* = 20), indicating that the intracellular Ca²⁺ stores responsible for the [Ca²⁺]_i oscillations were thapsigargin-sensitive (Fig. 2B). To examine whether the thapsigargin-sensitive intracellular Ca²⁺ store was releasable by IP₃ receptors or ryanodine receptors, cells were exposed to 75 μM 2-APB, 20 mM caffeine (IP₃ receptor antagonists), or 100 μM ryanodine (a ryanodine receptor antagonist), prior to the addition of 30 μM H₂O₂. As shown in Fig. 2C and supplemental Fig. S1, 2-APB (*n* = 17) and caffeine (*n* = 13) completely prevented the generation of [Ca²⁺]_i oscillations evoked by H₂O₂. In contrast to this, ryanodine failed to inhibit the H₂O₂-induced [Ca²⁺]_i oscillations (*n* = 15), although it effectively blocked the [Ca²⁺]_i increases induced by 500 μM caffeine, a ryanodine receptor agonist (*n* = 16; Fig. 2, D and E). These results suggest that thapsigargin-releasable, IP₃-sensitive Ca²⁺ stores are responsible for H₂O₂-induced Ca²⁺ mobilization.

Activation of PLC γ 1 Is Essential for the Generation of H₂O₂-induced [Ca²⁺]_i Oscillations—Because H₂O₂ has been shown to activate PLC γ 1 in some cell types (29, 30), we investigated whether PLC γ 1 was phos-

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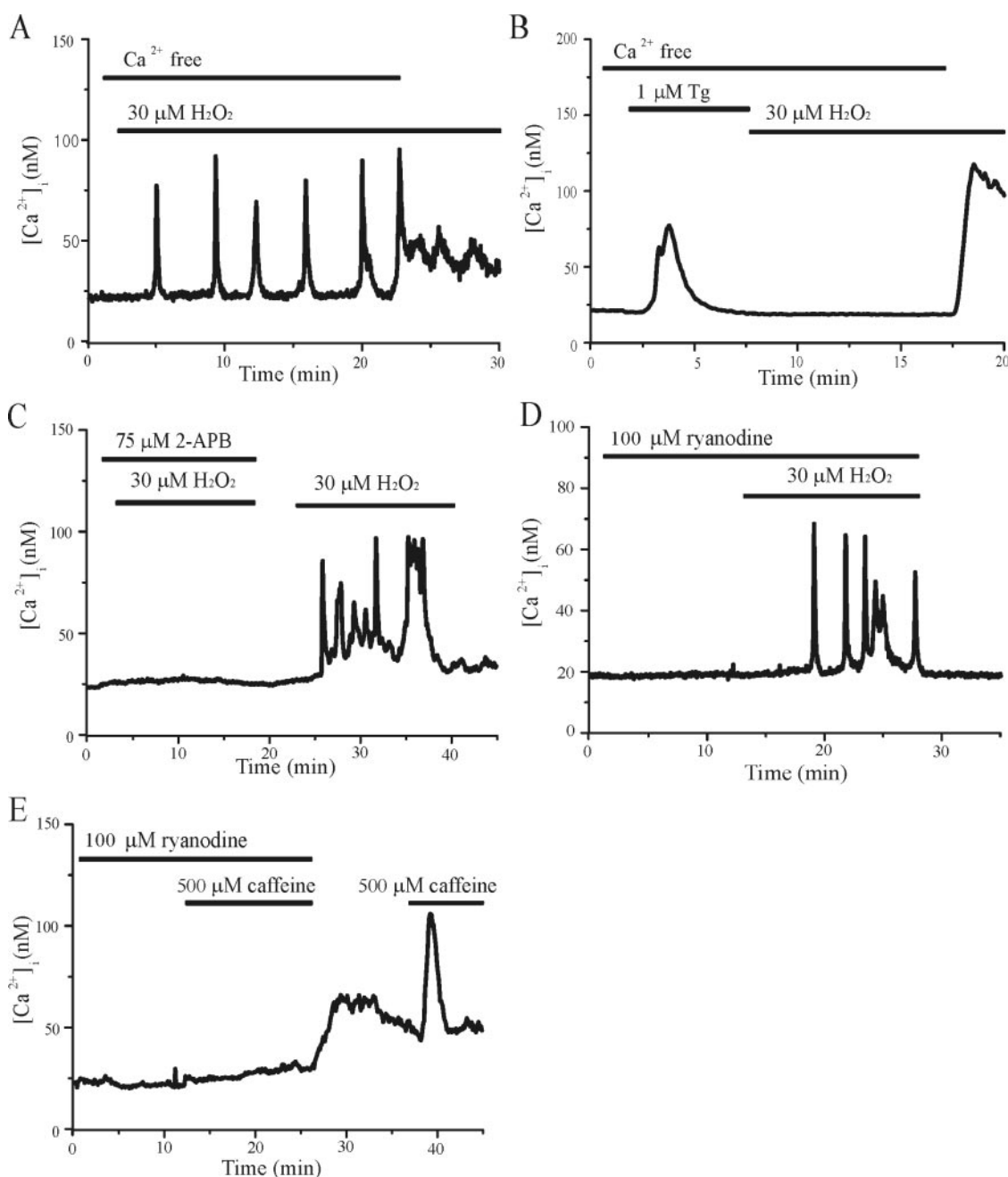


FIGURE 2. H₂O₂ mobilizes Ca²⁺ from thapsigargin-releasable, IP₃-sensitive Ca²⁺ stores in cultured rat cortical astrocytes. *A*, cells were exposed to 30 μM H₂O₂ in the absence of extracellular Ca²⁺ as indicated by the bars. *B*, cells were exposed to 1 μM thapsigargin (Tg) followed by 30 μM H₂O₂ in a nominally Ca²⁺-free solution. The absence of [Ca²⁺]_i increase after H₂O₂ application indicates that H₂O₂ mobilizes Ca²⁺ from a thapsigargin-sensitive Ca²⁺ store. *C* and *D*, H₂O₂-induced [Ca²⁺]_i oscillations were prevented by 75 μM 2-APB but not by 100 μM ryanodine. *E*, ryanodine blocked the [Ca²⁺]_i increases induced by 500 μM caffeine, a ryanodine receptor agonist. For each panel, the trace is representative of 15–20 cells in three or four independent experiments.

phorylated following H₂O₂ stimulation of cultured rat astrocytes. PLC γ 1 possesses three tyrosine residues, Tyr-771, Tyr-783, and Tyr-1254. Among them, Tyr-783 is known to be essential for IP₃ formation (31). Therefore, a phosphospecific tyrosine 783 antibody was used to detect the H₂O₂-induced phosphorylation of PLC γ 1. As shown in Fig. 3, *A* and *B*, exposure of the astrocytes to various concentrations of H₂O₂ for 10 min induced PLC γ 1 phosphorylation on tyrosine residue 783 in a dose-dependent manner ($n = 6$).

To clarify further the involvement of PLC γ 1 in the generation of [Ca²⁺]_i oscillations, we used the PLC inhibitor U73122 and as control its inactive analogue U73343. As shown in Fig. 3, *C* and *D*, 10 μM U73122, but not 10 μM U73343, prevented the H₂O₂-evoked [Ca²⁺]_i oscillations. The

frequencies of H₂O₂-induced [Ca²⁺]_i oscillations were 6.5 ± 1.2 peaks/20 min ($n = 14$) in the presence of U73343 and 0.8 ± 0.8 peaks/20 min ($n = 16$) in the presence of U73122. The effect of U73122 and U73343 on the H₂O₂-induced PLC γ 1 phosphorylation was also examined. As shown in Fig. 3, *E* and *F*, the phosphorylation of PLC γ 1 induced by H₂O₂ was inhibited by 10 μM U73122 but not by 10 μM U73343 ($n = 3$).

The involvement of PLC γ 1 was further investigated by using RNA interference (RNAi). As shown in Fig. 4, *A* and *B*, transfection of siRNA-PLC γ 1 suppressed the PLC γ 1 expression level, although that of PLC β 1 expression was not affected ($n = 5$). To examine the functional consequences of depletion of PLC γ 1 by RNAi, cells were transfected with pSUPER (empty vector) or siRNA-PLC γ 1 prior to measurement of

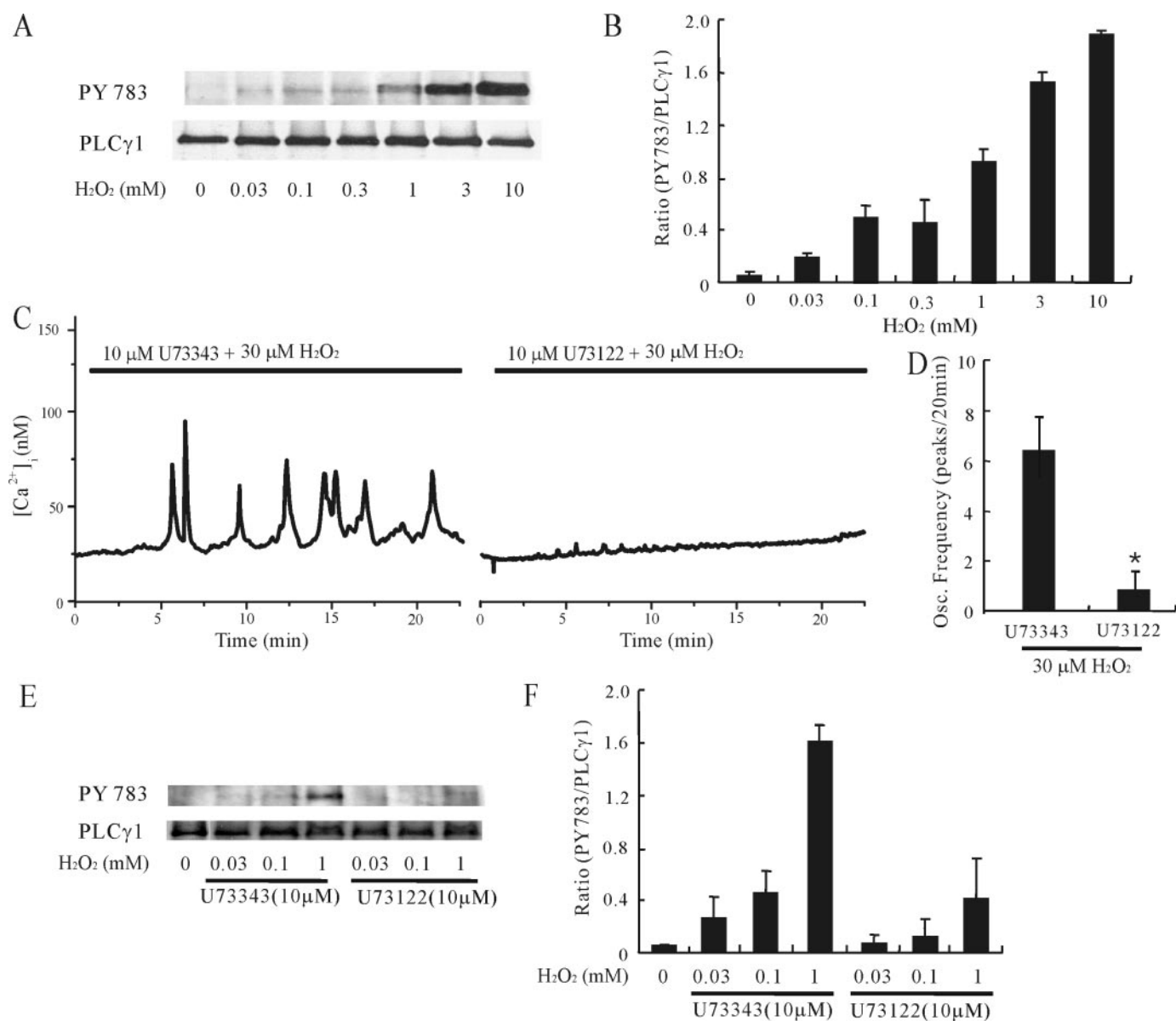


FIGURE 3. Activation of PLC γ 1 plays an essential role in H₂O₂-induced [Ca²⁺]_i oscillations in cultured rat cortical astrocytes. *A*, cells were treated with the indicated concentrations of H₂O₂ for 10 min, and lysates were subjected to immunoblot analysis with specific antibodies to phosphotyrosine (PY783) or PLC γ 1. *B*, data from six experiments were quantified, and the ratio (PY783/PLC γ 1) was calculated. *C*, cells were exposed to 30 μ M H₂O₂ in combination with U73343 or U73122 as indicated by the bars. *D*, quantitation of results in *C*. Oscillation frequency (peaks/20 min) was counted ($n = 14-16$). * indicates the difference of the oscillation frequencies between U73343- and U73122-treated groups ($p < 0.05$). *E*, cells were treated with the indicated concentrations of H₂O₂ in combination with 10 μ M U73343 or 10 μ M U73122 for 10 min, and lysates were subjected to immunoblot analysis with specific antibodies to phosphotyrosine (PY783) or PLC γ 1. *F*, data from three experiments were quantified, and the ratio (PY783/PLC γ 1) was calculated. Results are presented as mean \pm S.E.

[Ca²⁺]_i. As shown in Fig. 4, *C* and *D*, transfection with GFP and siRNA-PLC γ 1 resulted in the prevention of Ca²⁺ responses to H₂O₂ ($n = 10$). The frequencies of H₂O₂-induced [Ca²⁺]_i oscillations were 6.8 ± 0.26 peaks/20 min in GFP/PLC γ 1-negative cells and 0.5 ± 0.2 peaks/20 min in GFP/PLC γ 1-positive cells. Transfection with GFP and pSUPER had no effect on H₂O₂-induced [Ca²⁺]_i oscillations (data not shown).

To confirm whether the transfection of siRNA-PLC γ 1 had any effect on the Ca²⁺ response elicited by a PLC β -activating agonist, we stimulated the cells with histamine. As shown in Fig. 4, *E* and *F*, the cotransfection of GFP and siRNA-PLC γ 1 did not prevent the Ca²⁺ responses to histamine ($n = 6$). These results indicate that PLC γ 1 is necessary for the generation of [Ca²⁺]_i oscillations in response to H₂O₂.

In addition, we also observed that expression of the IP₃ sponge completely abrogated [Ca²⁺]_i oscillations in response to H₂O₂ ($n = 8$; see

Fig. 5, *A* and *B*), although the expression of low affinity IP₃ sponge did not prevent the H₂O₂-evoked [Ca²⁺]_i oscillations ($n = 6$; Fig. 5, *C* and *D*). This result strongly suggests that IP₃ production through the activation of PLC γ 1 is a critical step for the H₂O₂-induced generation of [Ca²⁺]_i oscillations.

Oxidation of a PLC γ 1-associated Signaling Component Is Responsible for H₂O₂-induced [Ca²⁺]_i Oscillations—To examine whether the H₂O₂-induced PLC γ 1 phosphorylation and [Ca²⁺]_i oscillations were attributed to the sulfhydryl oxidation-dependent mechanisms, we treated cells with 1 mM DTT, a sulfhydryl-reducing agent, 4 min prior to the addition of H₂O₂. As shown in Fig. 6, *A* and *B*, the phosphorylation of PLC γ 1 by H₂O₂ was prevented by pretreatment with 1 mM DTT ($n = 5$). Furthermore, the generation of [Ca²⁺]_i oscillations by 30 μ M H₂O₂ was also inhibited by 1 mM DTT ($n = 16$; Fig. 6*C*). These data suggest that

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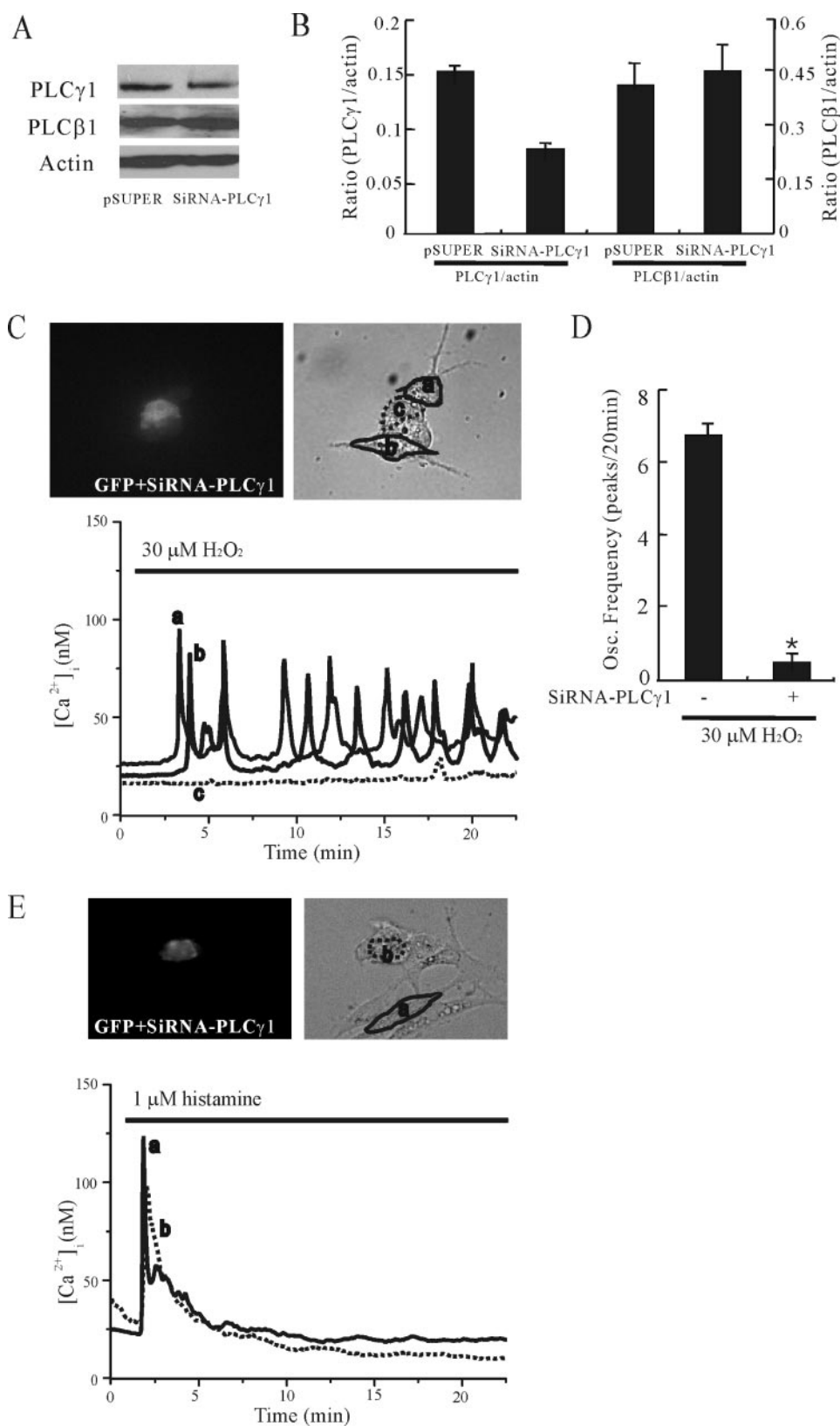


FIGURE 4. PLC γ 1 RNA interference results in the prevention of Ca²⁺ responses to H₂O₂ in cultured rat cortical astrocytes. *A*, Western blots of cell lysates transfected with pSUPER (empty vector) or siRNA for PLC γ 1 (*siRNA-PLC γ 1*). Gels were transferred and blotted with specific antibodies to PLC γ 1, PLC β 1, or actin. *B*, data from four experiments were quantified, and the ratios (*PLC γ 1/Actin* and *PLC β 1/Actin*) were calculated. *C* and *E*, cells were cotransfected with GFP and siRNA-PLC γ 1, and [Ca²⁺]_i was measured. Note that GFP- and siRNA-PLC γ 1-transfected cells (designated as *c* in *C*) exhibited a lack of Ca²⁺ response to 30 μ M H₂O₂ ($n = 8$; *C*), although GFP and siRNA-PLC γ 1 transfection (designated as *b* in *E*) did not affect the Ca²⁺ response to histamine ($n = 6$; *E*). *D*, the oscillation frequency (peaks/20 min) in cells transfected with or without siRNA-PLC γ 1 was counted during H₂O₂ stimulation. Results are means \pm S.E. * indicates the difference of the oscillation frequencies between siRNA-PLC γ 1-transfected and nontransfected groups ($p < 0.05$).

oxidation of a PLC γ 1-associated signaling component is responsible for the activation of PLC γ 1 and generation of [Ca²⁺]_i oscillations by H₂O₂.
H₂O₂ Might be Involved in the EGF-induced Activation of PLC γ 1 and Ca²⁺ Mobilization—Because it has been known that EGF elevates [Ca²⁺]_i and produces H₂O₂ in fibroblasts (32), we investigated whether

H₂O₂ was produced in response to EGF and if the endogenously produced H₂O₂ was involved in PLC γ 1 activation and [Ca²⁺]_i increases in cultured rat cortical astrocytes. As shown in Fig. 7, *A* and *B*, EGF at a concentration of 10 ng/ml induced an increase in DCF fluorescence intensity that was prevented by 5 mM NAC, indicating that EGF increased an

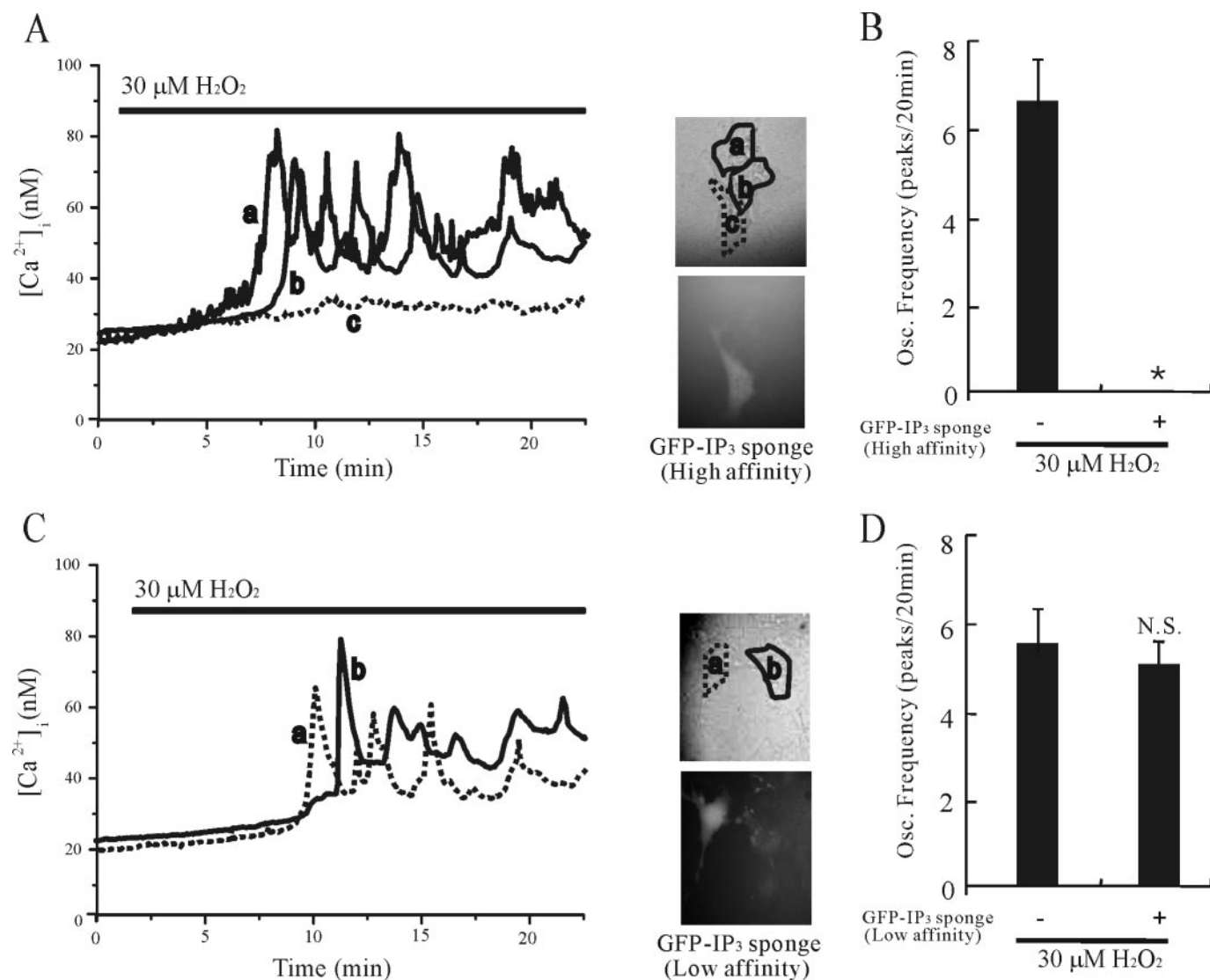


FIGURE 5. Expression of the IP₃ sponge prevents [Ca²⁺]_i oscillations evoked by H₂O₂ in cultured rat cortical astrocytes. Cells were transfected with GFP-tagged high affinity IP₃ sponge (A) or low affinity IP₃ sponge (C), and [Ca²⁺]_i was measured. Note that GFP-tagged high affinity IP₃ sponge-transfected cell (designated as c in A) exhibited the abolition of Ca²⁺ response to 30 μM H₂O₂, whereas GFP-tagged low affinity IP₃ sponge-transfected cell (designated as a in C) did not. The oscillation frequencies (peaks/20 min) in cells transfected with the high affinity (B) and low affinity IP₃ sponges (D) were counted during H₂O₂ stimulation in 8 and 6 independent experiments, respectively. Results are means ± S.E. * indicates the difference of the oscillation frequencies between the transfected and nontransfected groups ($p < 0.05$). N.S. indicates that there is no significant difference of the oscillation frequencies between the transfected and nontransfected groups ($p > 0.05$).

accumulation of ROS ($n = 5$). The DCF fluorescence intensity was also decreased by 10 μM DPI (an inhibitor of NADPH oxidase), suggesting that NADPH oxidase, at least in part, participated in the EGF-triggered generation of ROS ($n = 5$).

EGF induced a rapid transient peak increase in [Ca²⁺]_i that subsequently declined ($n = 15$; Fig. 8A). However, pretreatment with 5 mM NAC or 10 μM DPI attenuated the EGF-induced [Ca²⁺]_i increases, and removal of NAC and DPI in the continued presence of EGF increased [Ca²⁺]_i again ($n = 16-20$; Fig. 8, B and C). The effect of NAC and DPI on the EGF-induced activation of PLC γ 1 was also investigated. As shown in Fig. 8, D and E, the immediate strong activation of PLC γ 1 followed by a sustained weak activation was observed following EGF stimulation, but in the presence of 5 mM NAC or 10 μM DPI the activation of PLC γ 1 was greatly reduced ($n = 3$).

Because Prx II is a cellular peroxidase that eliminates endogenous H₂O₂ produced in response to growth factors such as EGF (33), we examined whether the overexpression of Prx II also attenuated EGF-induced [Ca²⁺]_i oscillations. As shown in Fig. 8, F and G, overexpression

of Prx II decreased the amplitude of EGF-induced [Ca²⁺]_i increase by about 57% ($n = 6$). These data suggested that H₂O₂ is generated by the activation of NADPH oxidase and is subsequently involved in the activation of PLC and the elevation of [Ca²⁺]_i during EGF stimulation in cultured rat astrocytes.

DISCUSSION

In this study, we report that exogenous addition of low concentrations of H₂O₂ triggers [Ca²⁺]_i oscillations through the activation of PLC γ 1 in cultured rat cortical astrocytes. H₂O₂-mediated elevation of cytosolic Ca²⁺ levels has been shown previously in various cell types (32–36). However, in many cases, [Ca²⁺]_i increases were induced by relatively high concentrations of H₂O₂, which are generally considered cytotoxic. The physiologically relevant concentration range of H₂O₂, which causes an acceleration of cellular functions in a variety of cell types, is considered 1–100 μM, although it depends on cell type (1, 37). In our system, we used 30 μM H₂O₂; it did not cause cell death (data not shown), and its effect on [Ca²⁺]_i was reversible, suggesting that 30 μM

Role of PLC γ 1 in H₂O₂-induced [Ca²⁺]_i Oscillations

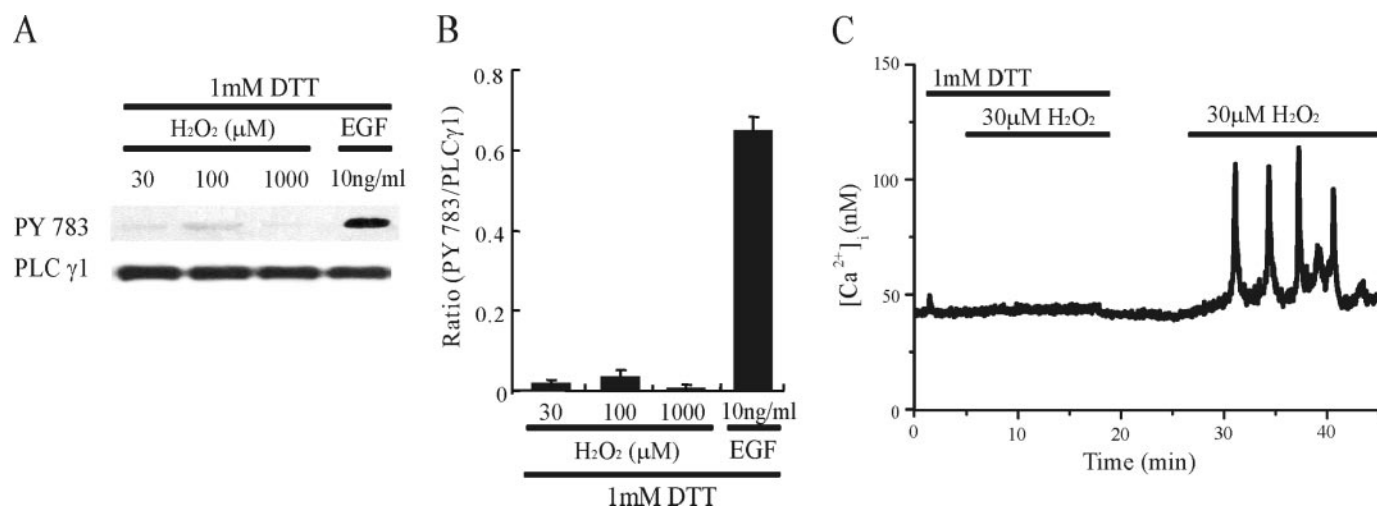


FIGURE 6. Oxidation of a PLC γ 1-associated signaling component is responsible for the H₂O₂-induced [Ca²⁺]_i oscillations in cultured rat cortical astrocytes. *A*, effect of DTT (1 mM), a sulfhydryl-reducing agent, on H₂O₂-induced PLC γ 1 phosphorylation. Cells were incubated with DTT for 4 min followed by an addition of indicated concentrations of H₂O₂ (1st to 3rd lanes) for 10 min or EGF (4th lane) for 2 min. Cell lysates were subjected to immunoblot analysis with specific antibodies to phosphotyrosine (PY783) or PLC γ 1. *B*, quantitation of results in *A*. Ratio (PY783/PLC γ 1) was calculated ($n = 5$). Results are presented as mean \pm S.E. Note that the dose-dependent increases in the ratio (PY783/PLC γ 1) by H₂O₂ shown in Fig. 3*B* were prevented by DTT. *C*, the Ca²⁺ responses of individual astrocytes to 30 μ M H₂O₂ in the presence or absence of 1 mM DTT are shown. Note that 1 mM DTT completely abolished H₂O₂-induced [Ca²⁺]_i oscillations. The result is representative of 16 cells in three independent experiments.

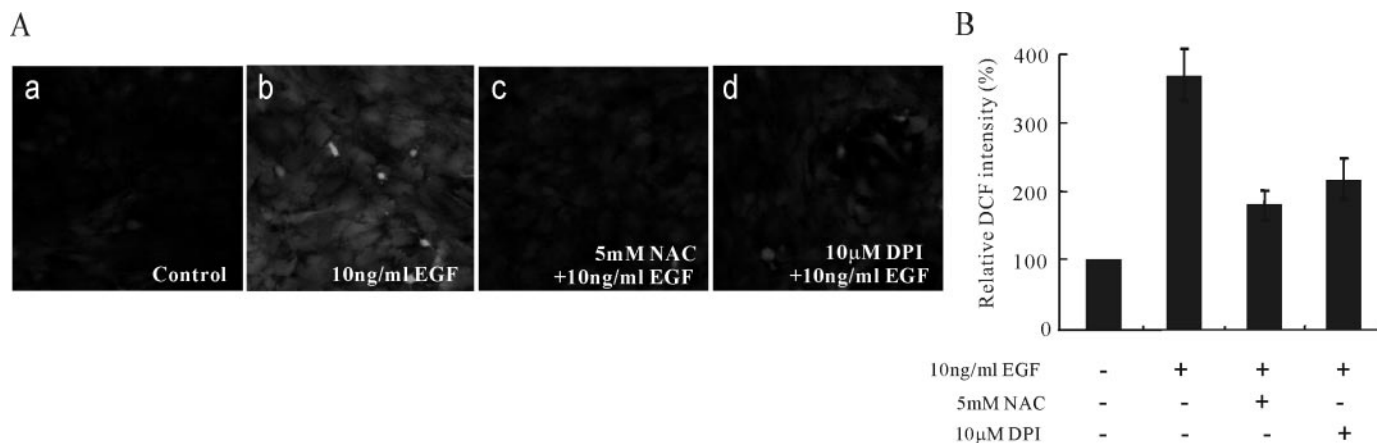


FIGURE 7. EGF produces ROS by the activation of NADPH oxidase in cultured rat cortical astrocytes. *A*, astrocytes were loaded with DCF for 5 min (*a-d*). Cells were treated with 5 mM NAC (*c*) or 10 μ M DPI (*d*) for 2 min followed by an addition of 10 ng/ml EGF (*b-d*) for 2 min. The fluorescence of DCF was subsequently visualized by confocal laser scanning microscopy. *B*, the DCF fluorescence was quantified, and the relative intensities were calculated by setting the fluorescence intensity of control cells to 100% ($n = 5$). Results are means \pm S.E.

H₂O₂ is close to the physiological concentration that modulates calcium signaling in astrocytes.

Cellular Ca²⁺ signals generally encode information in two different modes as follows: frequency-modulated and amplitude-modulated signals (38). Frequency-modulated Ca²⁺ signaling (*i.e.* [Ca²⁺]_i oscillations) is generally considered to have the highest fidelity, and many cells use this paradigm in response to low physiological concentrations of agonists (39). H₂O₂ has been shown to increase [Ca²⁺]_i in many cell types, but demonstrations of the generation of [Ca²⁺]_i oscillations have been rare. However, in this study we show that low concentrations of H₂O₂ generated [Ca²⁺]_i oscillations in astrocytes although high concentrations induced sustained increases in [Ca²⁺]_i. It is more reasonable for cells to use frequency-modulated Ca²⁺ signaling to avoid cell damage, especially when a prolonged period of Ca²⁺ signaling is necessary. In this regard, low concentration of H₂O₂ is considered to be a reliable intracellular messenger involved in Ca²⁺ signaling.

In most nonexcitable cells, such as astrocytes, both Ca²⁺ release from intracellular Ca²⁺ stores and Ca²⁺ influx through Ca²⁺ channels on the plasma membrane are necessary for the generation and maintenance of [Ca²⁺]_i oscillations (40). In the present study, we demonstrate that H₂O₂-

induced [Ca²⁺]_i oscillations were sustained in the absence of extracellular Ca²⁺, indicating that intracellular Ca²⁺ stores were primarily responsible for the generation of [Ca²⁺]_i oscillations. The two main intracellular organelles containing large amounts of Ca²⁺ are the endoplasmic reticulum and mitochondria (41). Previously, both of these Ca²⁺ stores were shown to be involved in H₂O₂-induced [Ca²⁺]_i increases (17). However, our data showed that depletion of intracellular Ca²⁺ stores with thapsigargin completely prevented the generation of H₂O₂-evoked [Ca²⁺]_i oscillations, suggesting that the thapsigargin-sensitive endoplasmic reticulum Ca²⁺ store was the source of [Ca²⁺]_i oscillations.

H₂O₂ was also reported to be involved in the mobilization of Ca²⁺ by activating intracellular Ca²⁺ channels, such as ryanodine receptors and IP₃ receptors (15, 16). The effect of ROS on ryanodine receptors has been well established. Sulfhydryl oxidation of ryanodine receptors has been reported to activate the channels (42, 43). However, in the present study, a high concentration of ryanodine (100 μ M), which blocked the Ca²⁺ mobilization induced by caffeine, a ryanodine receptor agonist, failed to prevent the H₂O₂-induced [Ca²⁺]_i oscillations. Instead, 2-APB and caffeine, IP₃-sensitive Ca²⁺ channel inhibitors, blocked the H₂O₂-induced [Ca²⁺]_i oscillations. 2-APB is known to have several cellular

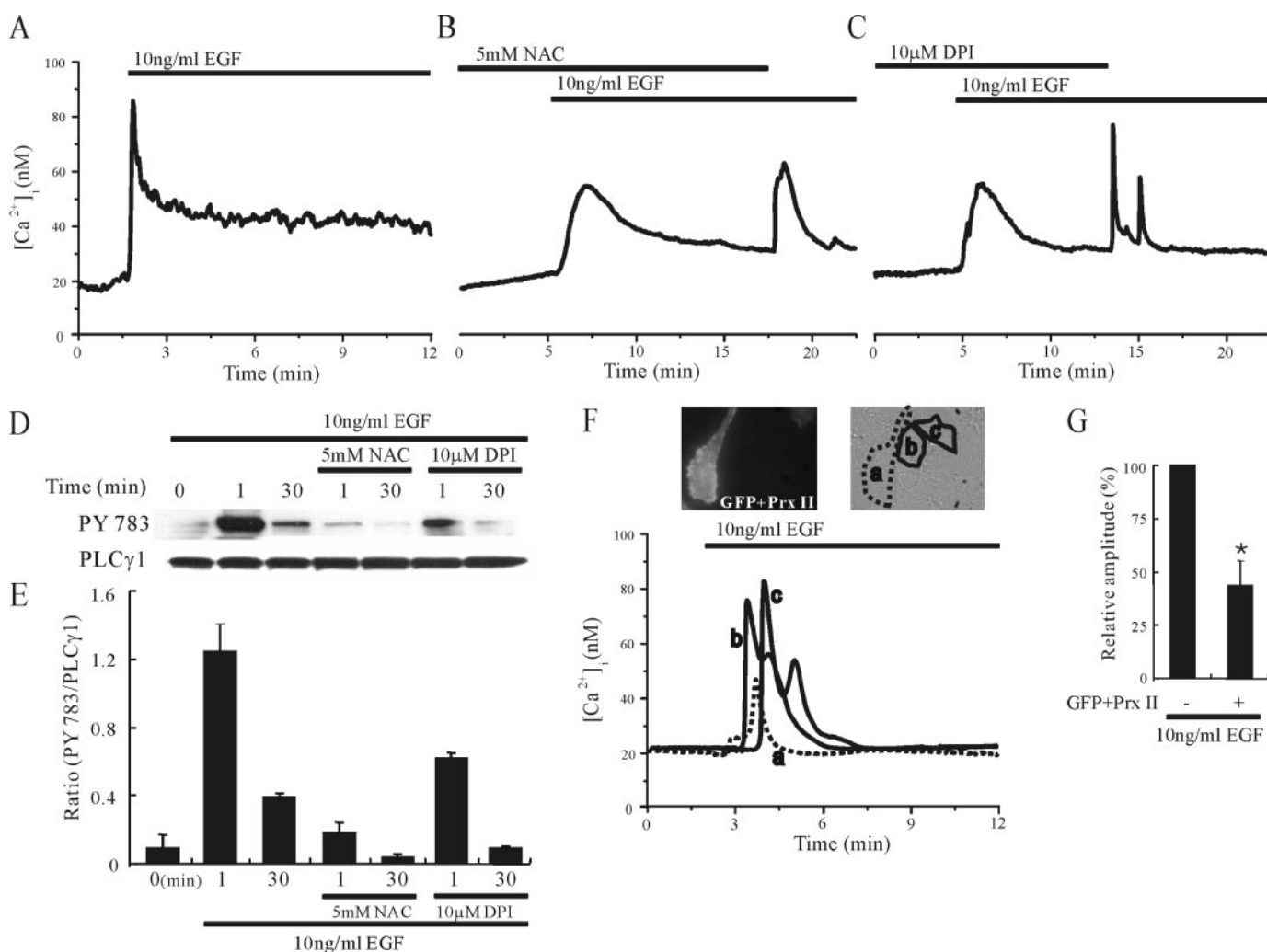


FIGURE 8. Endogenously produced H₂O₂ enhances EGF-induced PLC γ 1 activation and Ca²⁺ mobilization in cultured rat cortical astrocytes. The Ca²⁺ responses of single astrocytes to 10 ng/ml EGF in the absence (A) or presence of 5 mM NAC (B) or 10 μ M DPI (C) are denoted by the bars. Note that pretreatment with NAC or DPI suppressed Ca²⁺ responses to EGF, and the removal of NAC and DPI permitted the Ca²⁺ responses. Results are representative of 15–20 cells in three or four independent experiments. D, the effect of 5 mM NAC or 10 μ M DPI on 10 ng/ml EGF-induced PLC γ 1 phosphorylation is shown. Cells were incubated with or without NAC or DPI for 2 min, and then 10 ng/ml EGF was added for the indicated times. Cells were then lysed, and the lysates were subjected to immunoblot analysis with antibodies to phosphotyrosine (PY783) or PLC γ 1. E, quantitation of results in D. Ratio (PY783/PLC γ 1) was calculated ($n = 3$). F, cells were cotransfected with GFP and Prx II, which eliminates H₂O₂, and [Ca²⁺]_i was measured. Note that GFP- and Prx II-transfected cell (designated as a) exhibited reduced Ca²⁺ response to EGF. G, the amplitude of [Ca²⁺]_i increases (nM) in cells transfected with or without GFP and Prx II was measured during EGF stimulation in six independent experiments. Results are presented as mean \pm S.E. * indicates the difference of the amplitudes of [Ca²⁺]_i increases between the Prx II-transfected and nontransfected groups ($p < 0.05$).

targets; it blocks IP₃-sensitive Ca²⁺ channels, SERCA activity, and capacitative Ca²⁺ entry channels (44, 45). However, the inhibitory effect of 2-APB on [Ca²⁺]_i oscillations was unlikely to be due to the inhibition of SERCA, because the concentration of 2-APB we used in this study (75 μ M) was lower than the half-maximal inhibitory concentration for SERCA (91 μ M) (44). In addition, 75 μ M 2-APB did not show any evidence of [Ca²⁺]_i increase when applied to itself. This is in contrast to 1 μ M thapsigargin, a specific SERCA inhibitor, which induced a rapid increase in [Ca²⁺]_i as shown in Fig. 2B. Inhibition of SERCA has been shown to be associated with an increase in [Ca²⁺]_i in most cell types. Furthermore, it is unlikely that the effect of 2-APB on the [Ca²⁺]_i oscillations was because of inhibition of capacitative Ca²⁺ entry, because as shown for the experiments performed in the absence of extracellular Ca²⁺, Ca²⁺ entry is not required for the oscillations.

Caffeine also has several cellular targets. It can stimulate ryanodine receptors, inhibit both cAMP degradation and PLC activation, and prevent IP₃-sensitive Ca²⁺ channel opening. However, the only feature that caffeine and 2-APB share is their ability to antagonize IP₃-mediated

Ca²⁺ release. Therefore, although neither 2-APB nor caffeine are solely selective for IP₃-sensitive Ca²⁺ channels, when used judiciously these pharmacological agents can be used to reveal the specific involvement of IP₃ signaling. The results obtained using 2-APB and caffeine support the hypothesis that H₂O₂ induced [Ca²⁺]_i oscillations through activation of IP₃-sensitive Ca²⁺ channels.

H₂O₂ may activate signaling components responsible for IP₃ production. In some cell types, it has been reported that H₂O₂ induces an activation of PLC γ 1 (29, 30). PLC γ 1 is known to be recruited to the plasma membrane following activation of receptor tyrosine kinases and activated by a mechanism that relies on tyrosine phosphorylation (46). The phosphorylated PLC γ 1 catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to produce diacylglycerol and IP₃, leading to the activation of protein kinase C and increases in [Ca²⁺]_i, respectively (47). Recently, PLC γ 1 has been reported to be tyrosine-phosphorylated following H₂O₂ treatment and to protect cells from oxidant injury (18). However, the involvement of H₂O₂-induced PLC γ 1 activation in the generation of [Ca²⁺]_i oscillations, a typical form of physiological Ca²⁺

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responses, has not been explored. Here, we provide evidence supporting the key role of PLC γ 1 in the generation of [Ca²⁺]_i oscillations induced by low concentrations of H₂O₂. First, H₂O₂ induced dose-dependent phosphorylation of PLC γ 1, which was in agreement with the previous results reported by others (18, 29). Second, 30 μ M H₂O₂-induced [Ca²⁺]_i oscillations were almost completely prevented by U73122 but not by U73343. Third, RNAi against PLC γ 1 inhibited H₂O₂-induced [Ca²⁺]_i oscillations. Finally, expression of the IP₃ sponge completely abolished [Ca²⁺]_i oscillations in response to H₂O₂. These results suggest that the production of IP₃ following the activation of PLC γ 1 is essential for generation of H₂O₂-induced [Ca²⁺]_i oscillations in cultured rat cortical astrocytes. In addition to this, we showed that the H₂O₂-induced phosphorylation of PLC γ 1 and [Ca²⁺]_i oscillations were prevented by DTT, a sulfhydryl-reducing agent. Therefore, the oxidation of a PLC γ 1-associated signaling component appeared to be responsible for the H₂O₂-induced [Ca²⁺]_i oscillations.

Although the activation of PLC γ 1 was shown to play a critical role in the 30 μ M H₂O₂-evoked generation of [Ca²⁺]_i oscillations in our system, we do not rule out the possibility that H₂O₂ also increases the sensitivity of IP₃ receptors to IP₃ in this cell type. Previously, thimerosal, which catalyzes the oxidation of thiol groups, was reported to trigger [Ca²⁺]_i oscillations by increasing the affinity of IP₃ receptors for IP₃ (46, 49, 50). In addition, Hu *et al.* (16, 51) showed that NADPH oxidase-derived H₂O₂ increased the sensitivity of intracellular Ca²⁺ stores to IP₃ and played a critical role in generating [Ca²⁺]_i oscillations in human endothelial cells stimulated by histamine. Taken together, our study and those of Hu *et al.* (16, 51) imply that both PLC γ 1 activation and increased sensitivity of IP₃ receptors may contribute to the generation of [Ca²⁺]_i oscillations.

By having observed the stimulatory effect of exogenous H₂O₂ on intracellular Ca²⁺ signaling, we sought to determine whether H₂O₂ was produced by receptor stimulation and if endogenously generated H₂O₂ played a modulatory role in Ca²⁺ signaling in rat astrocytes. Stimulation of EGF receptors has been shown previously to induce both H₂O₂ production and [Ca²⁺]_i increases in fibroblasts (32). The EGF receptor belongs to a family of transmembrane receptors with intrinsic tyrosine kinase activity (52). The production of intracellular H₂O₂ in response to EGF was shown to require the activation of the Rac-NADPH oxidase signaling, whereas activation of PLC γ 1 was regarded to be critical for [Ca²⁺]_i increases (53–55). Although Rac was suggested to play a role in the EGF-induced Ca²⁺ signaling (32, 56), the role of H₂O₂ in PLC γ 1 activation during EGF stimulation has not been elucidated.

In this study, we showed that EGF receptor stimulation induced ROS production, PLC γ 1 activation, and [Ca²⁺]_i elevation, which were all attenuated by the pretreatment with NAC, an ROS scavenger, or DPI, an NADPH oxidase inhibitor. These results indicated that ROS produced via NADPH oxidase during EGF stimulation played a critical role in the enhancement and maintenance of PLC γ 1 and Ca²⁺ responses in rat cortical astrocytes. As far as we know, this is the first report to show the involvement of NADPH oxidase in EGF-mediated ROS generation and the regulatory role of endogenously produced ROS in PLC γ 1-activated Ca²⁺ signaling in astrocytes. Previous studies have revealed that the predominant member of ROS produced by EGF stimulation was H₂O₂, which played a key role in the EGF-induced protein tyrosine phosphorylation and [Ca²⁺]_i increases (4, 32). Furthermore, we found that overexpression of Prx II, which probably plays an important role in eliminating H₂O₂ generated in the cytosol, reduced the amplitude of [Ca²⁺]_i increase evoked by EGF. Therefore, it is likely that the major component of ROS, which is produced by EGF stimulation and responsible for PLC γ 1 activation and [Ca²⁺]_i elevation in our system, is H₂O₂.

Considering that the activation of EGF receptors stimulates the proliferation and differentiation of astrocytes (57, 58), the role of H₂O₂ in the regulation of Ca²⁺ signaling may be of physiological importance in this cell type.

We therefore conclude that physiologically relevant, low concentrations of H₂O₂ trigger the generation of [Ca²⁺]_i oscillations by activating PLC γ 1 through sulfhydryl oxidation-dependent mechanisms in cultured rat cortical astrocytes. Given that H₂O₂ is a small and diffusible molecule that is produced endogenously via NADPH oxidase during EGF receptor stimulation and is involved in the enhancement of Ca²⁺ signaling, H₂O₂ may be of physiological importance in regulating various cellular functions such as cell proliferation.

Acknowledgment—We thank Professor Michael Berridge for the valuable advice.

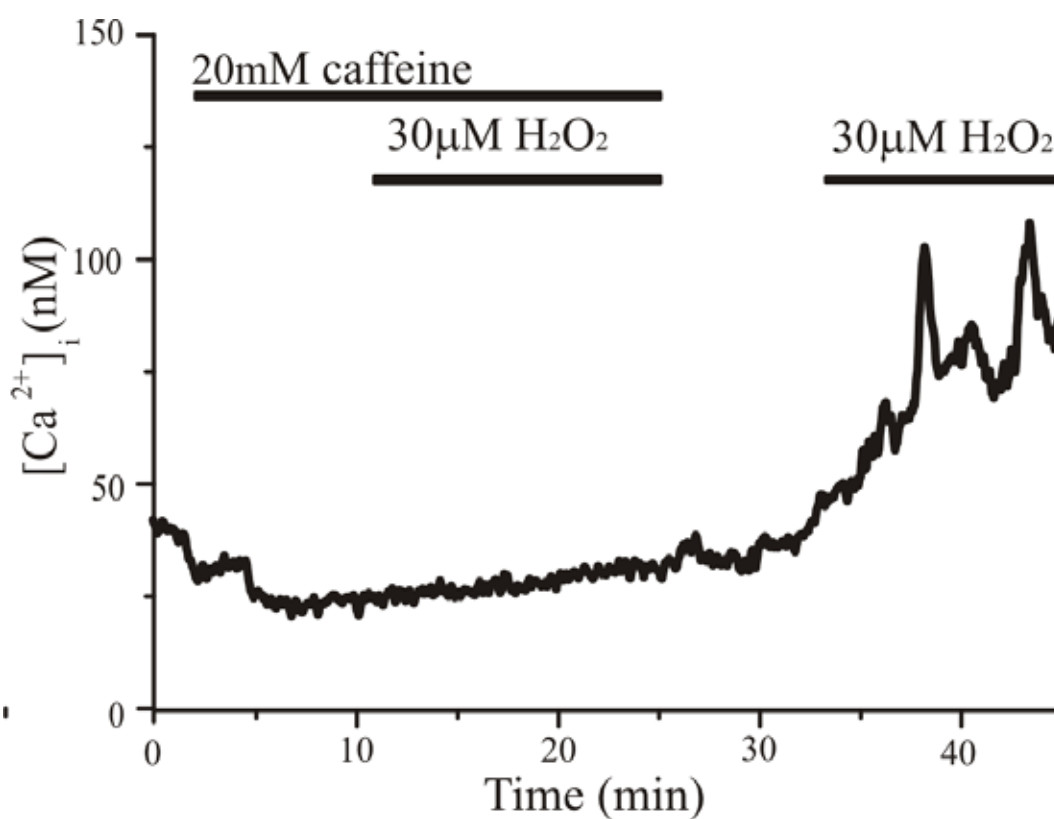
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Supplementary Figure

Fig. S1. Caffeine inhibited H₂O₂-induced [Ca²⁺]_i oscillations in cultured rat cortical astrocytes. Cells were exposed to 30 μM H₂O₂ in the presence of 20 mM caffeine as indicated by the bars. Note that pretreatment with 20 mM caffeine completely inhibited H₂O₂-induced [Ca²⁺]_i oscillations, whereas withdrawal of caffeine from the perfusate allowed the subsequent addition of H₂O₂ to induce [Ca²⁺]_i oscillations. The trace is representative of 13 cells in four independent experiments.



Critical Role of Phospholipase C γ 1 in the Generation of H₂O₂-evoked [Ca²⁺]_i Oscillations in Cultured Rat Cortical Astrocytes

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