Critical Role of Phospholipase Cγ1 in the Generation of H2O2-evoked [Ca2+]i Oscillations in Cultured Rat Cortical Astrocytes

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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) γ1 in a dose-dependent manner, and U73122, an inhibitor of PLC, completely abolished the H2O2-induced [Ca2+]i oscillations. In addition, RNA interference against PLCγ1 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 PLCγ1 phosphorylation were inhibited by pretreatment with dithiothreitol, a sulfhydryl-reducing agent. Finally, epidermal growth factor induced H2O2 production, PLCγ1 activation, and [Ca2+]i increases, which were attenuated by N-acetylcysteine and diphenyleneiodonium and by the overexpression of peroxiredoxin type II. Therefore, we conclude that low concentrations of exogenously applied H2O2 generate [Ca2+]i oscillations by activating PLCγ1 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), 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. 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-β1, 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) γ1 (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

The abbreviations used are: ROS, reactive oxygen species; PLCγ1, phospholipase Cγ1; [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; PLCβ1, phospholipase Cβ1, DTT, dithiothreitol; DPI, diphenyleneiodonium; NAC, N-acetylcysteine; DCF, 2′,7′-dichlorofluorescein diacetate; Pn 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 H_{2}O_{2}.

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 Ca^{2+} signaling to mediate a large spectrum of physiological responses. Elevation of [Ca^{2+}]_{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 Ca^{2+} 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 H_{2}O_{2} and cell survival in this cell type (25). Given the widespread involvement of H_{2}O_{2} in modulating Ca^{2+} signaling cascades, it is tempting to speculate that astrocytes may also use redox signaling to modify their Ca^{2+} signaling.

Therefore, in the present study, we sought to investigate the roles of H_{2}O_{2} in Ca^{2+} signaling in cultured rat astrocytes. Our results indicate that a low, physiologically relevant concentration of H_{2}O_{2} (30 μM) induces [Ca^{2+}]_{i} oscillations in a PLCγ1- and IP_{3}-dependent manner. In addition, H_{2}O_{2} produced endogenously by EGF receptor stimulation is involved in the modulation of Ca^{2+} signaling in rat astrocytes.

EXPERIMENTAL PROCEDURES

Materials—H_{2}O_{2}, dithiothreitol (DTT), thapsigargin, 2-aminoethoxydiphenylborate (2-APB), ryanodine, N-acetylcysteine (NAC), diphenyleenedione (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-aceotxymethyl ester was purchased from Teflabs (Austin, TX). All other chemicals were of reagent grade. The polyclonal antibody against PLCγ1 and monoclonal antibody against phosphotyrosine (PY783) of PLCγ1 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-PLCβ1 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% CO_{2} and 95% air for 2–3 weeks. For [Ca^{2+}]_{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 μg/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 IP_{3} Sponge and Peroxiredoxin Type II (Prrx II)—Astrocytes were transiently transfected with a green fluorescent protein (GFP)-tagged high affinity (R441Q) or low affinity (K508A) IP_{3}-sequestering sponge (26), or were cotransfected with Prrx II (1 μg/ml, kindly provided by Professor S. W. Kang, Ewha Womans University, Seoul, Korea) and eGFP-N1 (1.2 μg/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% CO_{2} 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-PLCγ1—Construction of a small interfering RNA (siRNA) for PLCγ1 (siRNA-PLCγ1) was described before (27). The pSUPER vector for siRNA was purchased from OligoEngine (Seattle, WA). Cells were cotransfected with siRNA-PLCγ1 (1 μg/ml) and eGFP-N1 (1.2 μg/ml) using Lipofectamine 2000 reagent and cultured for 48 h in serum-free MEM. Depletion of endogenous PLCγ1 by siRNA was confirmed by immunoblot or GFP fluorescence.

Western Blot Analysis—Astrocytes transfected with or without siRNA-PLCγ1 and eGFP-N1 were stimulated with H_{2}O_{2} or EGF for the indicated times in the physiological salt solutions (PSSs) containing 140 mM NaCl, 5 mM KCl, 1 mM MgCl_{2}, 1 mM CaCl_{2}, 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 phenylmethanesulfonyl fluoride, 1 μg/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 PLCγ1, phosphospecific tyrosine 783, PLCβ1, and actin, and the proteins were detected by ECL (Amer-Sham Biosciences). The intensity of bands was quantified using Meta-Morph Analysis System (Universal Imaging Co., Downingtown, PA).

[Ca^{2+}]_{i}, Measurements—For [Ca^{2+}]_{i}, measurements, attached cells were loaded with fura-2 by incubation with 3.5 μM fura-2-acetoxymethyl ester in PSS equilibrated with 100% O_{2} 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 Ca^{2+}-free solutions, CaCl_{2} 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 [Ca^{2+}]_{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 μg/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 ± 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

H_{2}O_{2} Mobilizes Ca^{2+} in Cultured Rat Cortical Astrocytes in a Concentration-dependent Manner—The effect of H_{2}O_{2} on Ca^{2+} mobilization was examined in fura-2-loaded cultured rat astrocytes. Exposure of the cells to H_{2}O_{2} at concentrations lower than 3 μM failed to increase [Ca^{2+}]_{i}, at least for 20 min (Fig. 1A). However, 10 μM H_{2}O_{2} was shown to induce [Ca^{2+}]_{i}, oscillations in 34.7 ± 8.4% of the 18 tested cells, and the
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A

1 µM H₂O₂

[Ca²⁺] (nM)

Time (min)

0

20

40

60

80

100

B

30 µM H₂O₂

[Ca²⁺] (nM)

Time (min)

0

20

40

60

80

100

C

1 mM H₂O₂

[Ca²⁺] (nM)

Time (min)

0

20

40

60

80

100

D

No. of Responding Cells (%)

0

10

20

30

40

50

60

70

80

90

100

H₂O₂ (µM)

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⁺], 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⁺], whereas doses higher than 300 µM caused spike and plateau type [Ca²⁺] 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⁺] 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⁺] 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⁺] oscillations persisted in the absence of extracellular Ca²⁺ (n = 18), suggesting that intracellular Ca²⁺ stores were the main source for H₂O₂-induced [Ca²⁺] oscillations. Depletion of the intracellular Ca²⁺ stores with thapsigargin, a specific inhibitor of SERCA, prevented H₂O₂-induced [Ca²⁺] oscillations (n = 20), indicating that the intracellular Ca²⁺ stores responsible for the [Ca²⁺] 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⁺] oscillations evoked by H₂O₂. In contrast to this, ryanodine failed to inhibit the H₂O₂-induced [Ca²⁺] oscillations (n = 15), although it effectively blocked the [Ca²⁺] 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 PLCγ1 Is Essential for the Generation of H₂O₂-induced [Ca²⁺] Oscillations—Because H₂O₂ has been shown to activate PLCγ1 in some cell types (29, 30), we investigated whether PLCγ1 was phos-
phorylated following H$_2$O$_2$ stimulation of cultured rat astrocytes. PLC$\gamma_1$ possesses three tyrosine residues, Tyr-771, Tyr-783, and Tyr-1254. Among them, Tyr-783 is known to be essential for IP$_3$ formation (31). Therefore, a phosphospecific tyrosine 783 antibody was used to detect the H$_2$O$_2$-induced phosphorylation of PLC$\gamma_1$. As shown in Fig. 3, A and B, exposure of the astrocytes to various concentrations of H$_2$O$_2$ for 10 min induced PLC$\gamma_1$ phosphorylation on tyrosine residue 783 in a dose-dependent manner ($n=6$).

To clarify further the involvement of PLC$\gamma_1$ in the generation of [Ca$^{2+}$]$_i$ oscillations, we used the PLC inhibitor U73122 and as control its inactive analogue U73343. As shown in Fig. 3, C and D, H$_2$O$_2$-induced [Ca$^{2+}$]$_i$ oscillations were prevented by 75 $\mu$M 2-APB but not by 100 $\mu$M ryanodine. E, ryanodine blocked the [Ca$^{2+}$]$_i$ increases induced by 500 $\mu$M caffeine, a ryanodine receptor agonist. For each panel, the trace is representative of 15–20 cells in three or four independent experiments.

The role of PLC$\gamma_1$ in H$_2$O$_2$-induced [Ca$^{2+}$]$_i$ Oscillations

H$_2$O$_2$ mobilizes Ca$^{2+}$ from thapsigargin-releasable, IP$_3$-sensitive Ca$^{2+}$ stores in cultured rat cortical astrocytes. A, cells were exposed to 30 $\mu$M H$_2$O$_2$ in the absence of extracellular Ca$^{2+}$ as indicated by the bars. B, cells were exposed to 1 $\mu$M thapsigargin (Tg) followed by 30 $\mu$M H$_2$O$_2$ in a nominally Ca$^{2+}$-free solution. The absence of [Ca$^{2+}$]$_i$ increase after H$_2$O$_2$ application indicates that H$_2$O$_2$ mobilizes Ca$^{2+}$ from a thapsigargin-sensitive Ca$^{2+}$ store. C and D, H$_2$O$_2$-induced [Ca$^{2+}$]$_i$ oscillations were prevented by 75 $\mu$M 2-APB but not by 100 $\mu$M ryanodine. E, ryanodine blocked the [Ca$^{2+}$]$_i$ increases induced by 500 $\mu$M caffeine, a ryanodine receptor agonist. For each panel, the trace is representative of 15–20 cells in three or four independent experiments.
As shown in Fig. 4, C and D, transfection with GFP and siRNA-PLCγ1 resulted in the prevention of Ca\(^{2+}\) responses to H\(_2\)O\(_2\) (n = 10). The frequencies of H\(_2\)O\(_2\)-induced [Ca\(^{2+}\)]\(_i\) oscillations were 6.8 ± 0.26 peaks/20 min in GFP/PLCγ1-negative cells and 0.5 ± 0.2 peaks/20 min in GFP/PLCγ1-positive cells. Transfection with GFP and pSUPER had no effect on H\(_2\)O\(_2\)-induced [Ca\(^{2+}\)]\(_i\) oscillations (data not shown).

To confirm whether the transfection of siRNA-PLCγ1 had any effect on the Ca\(^{2+}\) response elicited by a PLC-activating agonist, we stimulated the cells with histamine. As shown in Fig. 4, E and F, the cotransfection of GFP and siRNA-PLCγ1 did not prevent the Ca\(^{2+}\) responses to histamine (n = 6). These results indicate that PLCγ1 is necessary for the generation of [Ca\(^{2+}\)]\(_i\) oscillations in response to H\(_2\)O\(_2\).

In addition, we also observed that the expression of the IP\(_3\) sponge completely abrogated [Ca\(^{2+}\)]\(_i\) oscillations in response to H\(_2\)O\(_2\) (n = 8; see Fig. 5, A and B). Although the expression of low affinity IP\(_3\) sponge did not prevent the H\(_2\)O\(_2\)-evoked [Ca\(^{2+}\)] oscillations (n = 6; Fig. 5, C and D), this result strongly suggests that IP\(_3\) production through the activation of PLCγ1 is a critical step for the H\(_2\)O\(_2\)-induced generation of [Ca\(^{2+}\)] oscillations.

**Oxidation of a PLCγ1-associated Signaling Component Is Responsible for H\(_2\)O\(_2\)-induced [Ca\(^{2+}\)] Oscillations.**—To examine whether the H\(_2\)O\(_2\)-induced PLCγ1 phosphorylation and [Ca\(^{2+}\)] 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 H\(_2\)O\(_2\). As shown in Fig. 6, A and B, the phosphorylation of PLCγ1 by H\(_2\)O\(_2\) was prevented by pretreatment with 1 mM DTT (n = 5). Furthermore, the generation of [Ca\(^{2+}\)] oscillations by 30 μM H\(_2\)O\(_2\) was also inhibited by 1 mM DTT (n = 16; Fig. 6C). These data suggest that...
Oxidation of a PLCγ1-associated signaling component is responsible for the activation of PLCγ1 and generation of \([Ca^{2+}]_i\) oscillations by \(H_2O_2\).

\(H_2O_2\) Might be Involved in the EGF-induced Activation of PLCγ1 and \([Ca^{2+}]_i\) Mobilization—Because it has been known that EGF elevates \([Ca^{2+}]_i\) and produces \(H_2O_2\) in fibroblasts (32), we investigated whether \(H_2O_2\) was produced in response to EGF and if the endogenously produced \(H_2O_2\) was involved in PLCγ1 activation and \([Ca^{2+}]_i\) 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
accumulation of ROS \((n = 5)\). The DCF fluorescence intensity was also decreased by \(10 \mu M\) 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 \([Ca^{2+}]_i\) that subsequently declined \((n = 15; \text{Fig. 8A})\). However, pretreatment with \(5 mM\) NAC or \(10 \mu M\) DPI attenuated the EGF-induced \([Ca^{2+}]_i\) increases, and removal of NAC and DPI in the continued presence of EGF increased \([Ca^{2+}]_i\), again \((n = 16–20; \text{Fig. 8, B and C})\). The effect of NAC and DPI on the EGF-induced activation of PLC\(\gamma_1\) was also investigated. As shown in Fig. 8, D and E, the immediate strong activation of PLC\(\gamma_1\) followed by a sustained weak activation was observed following EGF stimulation, but in the presence of \(5 mM\) NAC or \(10 \mu M\) DPI the activation of PLC\(\gamma_1\) was greatly reduced \((n = 3)\).

Because Prx II is a cellular peroxidase that eliminates endogenous \(H_2O_2\) produced in response to growth factors such as EGF \((33)\), we examined whether the overexpression of Prx II also attenuated EGF-induced \([Ca^{2+}]_i\) oscillations. As shown in Fig. 8, F and G, overexpression of Prx II decreased the amplitude of EGF-induced \([Ca^{2+}]_i\), increase by about \(57\% (n = 6)\). These data suggested that \(H_2O_2\) is generated by the activation of NADPH oxidase and is subsequently involved in the activation of PLC and the elevation of \([Ca^{2+}]_i\), during EGF stimulation in cultured rat astrocytes.

**DISCUSSION**

In this study, we report that exogenous addition of low concentrations of \(H_2O_2\) triggers \([Ca^{2+}]_i\) oscillations through the activation of PLC\(\gamma_1\) in cultured rat cortical astrocytes. \(H_2O_2\)-mediated elevation of cytosolic \(Ca^{2+}\) levels has been shown previously in various cell types \((32–36)\). However, in many cases, \([Ca^{2+}]_i\) increases were induced by relatively high concentrations of \(H_2O_2\), which are generally considered cytotoxic. The physiologically relevant concentration range of \(H_2O_2\), which causes an acceleration of cellular functions in a variety of cell types, is considered \(1–100 \mu M\), although it depends on cell type \((1, 37)\).

In our system, we used \(30 \mu M\) \(H_2O_2\); it did not cause cell death \(\text{(data not shown)}\), and its effect on \([Ca^{2+}]_i\) was reversible, suggesting that \(30 \mu M\)
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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 have been reported to activate the channels (42, 43). However, in the present study, a high concentration of ryanodine (100 μM), which blocked the thapsigargin-sensitive endoplasmic reticulum Ca²⁺ store, was not effective for the generation of H₂O₂-evoked [Ca²⁺]ᵢ oscillations. Instead, 2-APB was effective for the generation of thapsigargin completely prevented the generation of H₂O₂-evoked [Ca²⁺]ᵢ oscillations, suggesting that the thapsigargin-sensitive endoplasmic reticulum Ca²⁺ store was the source for [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. Sulphydryl oxidation of ryanodine receptors has been reported to activate the channels (42, 43). However, in the present study, a high concentration of ryanodine (100 μM), 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...
targets; it blocks IP$_3$-sensitive Ca$^{2+}$ channels, SERCA activity, and capacitative Ca$^{2+}$ entry channels (44, 45). However, the inhibitory effect of 2-APB on [Ca$^{2+}$]$_i$ oscillations was unlikely to be due to the inhibition of SERCA, because the concentration of 2-APB we used in this study (75 μM) was lower than the half-maximal inhibitory concentration for SERCA (91 μM) (44). In addition, 75 μM 2-APB did not show any evidence of [Ca$^{2+}$]$_i$ increase when applied to itself. This is in contrast to 1 μM thapsigargin, a specific SERCA inhibitor, which induced a rapid increase in [Ca$^{2+}$]$_i$, as shown in Fig. 2B. Inhibition of SERCA has been shown to be associated with an increase in [Ca$^{2+}$]$_i$, in most cell types. Furthermore, it is unlikely that the effect of 2-APB on the [Ca$^{2+}$]$_i$ oscillations was because of inhibition of capacitative Ca$^{2+}$ entry, because as shown for the experiments performed in the absence of extracellular Ca$^{2+}$, Ca$^{2+}$ 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 IP$_3$-sensitive Ca$^{2+}$ channel opening. However, the only feature that caffeine and 2-APB share is their ability to antagonize IP$_3$-mediated Ca$^{2+}$ release. Therefore, although neither 2-APB nor caffeine are solely selective for IP$_3$-sensitive Ca$^{2+}$ channels, when used judiciously these pharmacological agents can be used to reveal the specific involvement of IP$_3$ signaling. The results obtained using 2-APB and caffeine support the hypothesis that H$_2$O$_2$ induced [Ca$^{2+}$]$_i$ oscillations through activation of IP$_3$-sensitive Ca$^{2+}$ channels.

H$_2$O$_2$ may activate signaling components responsible for IP$_3$ production. In some cell types, it has been reported that H$_2$O$_2$ induces an activation of PLCγ1 (29, 30). PLCγ1 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 PLCγ1 catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to produce diacylglycerol and IP$_3$, leading to the activation of protein kinase C and increases in [Ca$^{2+}$]$_i$ (47). Recently, PLCγ1 has been reported to be tyrosine-phosphorylated following H$_2$O$_2$ treatment and to protect cells from oxidant injury (18). However, the involvement of H$_2$O$_2$-induced PLCγ1 activation in the generation of [Ca$^{2+}$]$_i$ oscillations, a typical form of physiological Ca$^{2+}$

FIGURE 8. Endogenously produced H$_2$O$_2$ enhances EGF-induced PLCγ1 activation and Ca$^{2+}$ mobilization in cultured rat cortical astrocytes. The Ca$^{2+}$ responses of single astrocytes to 10 ng/ml EGF in the absence (A) or presence of 5 mM NAC (B) or 10 μM DPI (C) are denoted by the bars. Note that pretreatment with NAC or DPI suppressed Ca$^{2+}$ responses to EGF, and the removal of NAC and DPI permitted the Ca$^{2+}$ responses. Results are representative of 15–20 cells in three or four independent experiments. D, the effect of 5 mM NAC or 10 μM DPI on 10 ng/ml EGF-induced PLCγ1 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 PLCγ1. E, quantitation of results in D. Ratio (PY783/PLCγ1) was calculated (n = 3). F, cells were cotransfected with GFP and Prx II, which eliminates H$_2$O$_2$, and [Ca$^{2+}$]$_i$ 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 ± S.E. * indicates the difference of the amplitudes of [Ca$^{2+}$]$_i$ increases between the Prx II-transfected and nontransfected groups (p < 0.05).
Role of PLCγ1 in H2O2-induced [Ca2+]i Oscillations

responses, has not been explored. Here, we provide evidence supporting the key role of PLCγ1 in the generation of [Ca2+]i oscillations induced by low concentrations of H2O2. First, H2O2 induced dose-dependent phosphorylation of PLCγ1, which was in agreement with the previous results reported by others (18, 29). Second, 30 μM H2O2-induced [Ca2+]i oscillations were almost completely prevented by U73122 but not by U73343. Third, RNAi against PLCγ1 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 PLCγ1 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 PLCγ1 and [Ca2+]i oscillations were prevented by DTT, a sulfhydryl-reducing agent. Therefore, the oxidation of a PLCγ1-associated signaling component appeared to be responsible for the H2O2-induced [Ca2+]i oscillations.

Although the activation of PLCγ1 was shown to play a critical role in the 30 μM 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 played a modulatory role in Ca2+ signaling, whereas activation of PLCγ1 was regarded to be critical for [Ca2+]i oscillations (23, 24). The production of intracellular H2O2 in response to EGF was shown to require the activation of the Rac-NADPH oxidase (20, 21) and the key role of PLCγ1 was considered to be physiological importance in this cell type.

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 PLCγ1 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.

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References

1. Droge, W. (2002) Physiol. Rev. 82, 47–95
2. Ohba, M., Shibamura, M., Kuroki, T., and Nose, K. (1994) J. Cell Biol. 126, 1079–1088
3. Sundaresan, M., Yu, Z. X., Ferrans, V. J., Irani, K., and Finkel, T. (1995) Science 270, 296–299
4. Bae, S. Y., Kang, S. W., Seo, M. S., Baines, I. C., Tekle, E., Chock, P. B., and Rhee, S. G. (1997) J. Biol. Chem. 272, 217–221
5. Bae, S. Y., Sung, J. Y., Kim, O. S., Kim, Y. J., Hur, K. C., Kazlauskas, A., and Rhee, S. G. (2000) J. Biol. Chem. 275, 10527–10531
6. Sober, C. G., Heistad, D. D., and Faraci, F. M. (1997) Stroke 28, 2290–2294
7. Pignattiello, P., Pulcinelli, F. M., Lentil, L., Gazzaniga, P. P., and Voil, F. (1998) Blood 91, 484–490
8. Varela, D., Simon, F., Riveros, A., Jorgensen, F., and Stutzin, A. (2004) J. Biol. Chem. 279, 13301–13304
9. Tournaire, C., Thomas, G., Pierre, J., Jacquemin, C., Pierre, M., and Saunier, B. (1997) Eur. J. Biochem. 244, 587–595
10. Akashi, T., Nakazawa, K., Sato, K., Saito, H., Ohno, Y., and Ito, Y. (2004) Neurosci. Lett. 356, 25–28
11. Berridge, M. J., Lipp, P., and Bootman, M. D. (2000) Nat. Rev. Mol. Cell Biol. 1, 11–21
12. Clapham, D. E. (1995) Cell 80, 259–268
13. Putney, J. W., Jr., Broad, L. M., Braun, F. J., Lievremont, J. P., and Bird, G. S. (2001) J. Cell Sci. 114, 2223–2229
14. Wehage, E., Eisfeld, J., Heiner, I., Jungling, E., Zitt, C., and Luckhoff, A. (2002) J. Biol. Chem. 277, 23150–23156
15. Favero, T. G., Zable, A. C., and Abramson, J. J. (1995) J. Biol. Chem. 270, 25557–25563
16. Hu, Q., Zheng, G., Zweier, J. L., Deshpande, S., Irani, K., and Ziegelstein, R. C. (2000) J. Biol. Chem. 275, 15749–15757
17. Redondo, P. C., Salido, G. M., Rosado, J. A., and Pariente, J. A. (2004) Biochim. Biophys. Acta 1684, 491–502
18. Wang, X. T., McCullough, K. D., Wang, X. J., Carpenter, G., and Holbrook, N. J. (2001) J. Biol. Chem. 276, 28364–28371
19. Servitj, M., Masgrau, R., Pardo, R., Sarri, E., and Picatoste, F. (2000) J. Neurochem. 75, 788–794
20. Hansson, E., and Ronnback, L. (2003) FASEB J. 17, 341–348
21. Newman, E. A. (2003) Trends Neurosci. 26, 536–542
22. Tanaka, J., Toki, K., Zhang, B., Ishihara, K., Sakanaika, M., and Maeda, N. (1999) GLIA 28, 85–96
23. Wang, X. F., and Cynader, M. S. (2000) J. Neurochem. 74, 1434–1442
24. Takuma, K., Baba, A., and Matsuda, T. (2004) Prog. Neurobiol. 72, 111–127
25. Liu, Q., Kang, J. H., and Zheng, R. L. (2005) Cell Biochem. Funct. 23, 93–100
26. Uchiyama, T., Yoshikawa, F., Hishida, A., Furuichi, T., and Mikoshiba, K. (2002) J. Biol. Chem. 277, 8106–8113
27. Lee, I. H., You, J. O., Ha, K. S., Bae, D. S., Suh, P. G., Rhee, S. G., and Bae, Y. S. (2004) J. Biol. Chem. 279, 26645–26653
28. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
29. Qin, S., Inazu, T., and Yamamura, H. (1995) Biochem. J. 308, 347–352
30. Toomre, D. K., Sloan, K. J., Iwasaki, T., and Fukami, Y. (2002) Cell Calcium 32, 11–20
31. Kim, H. K., Kim, J. W., Ziberstein, A., Margolis, B., Kim, J. G., Schlessinger, J., and Rhee, S. G. (1991) Cell 65, 435–441
32. Lee, Z. W., Kweon, S. M., Kim, S. J., Kim, J. H., Cheong, C., Park, Y. M., and Ha, K. S. (2000) Cell. Signal. 12, 91–98
33. Choi, M. H., Lee, I. K., Kim, G. W., Kim, B. U., Han, Y. H., Yu, D. Y., Park, H. S., Kim,
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K. Y., Lee, J. S., Choi, C., Bae, Y. S., Lee, B. I., Rhee, S. G., and Kang, S. W. (2005) Nature 435, 347–353
34. Pariente, J. A., Camello, C., Camello, P. J., and Salido, G. M. (2001) J. Membr. Biol. 179, 27–35
35. Smith, M. A., Herson, P. S., Lee, K., Pinnock, R. D., and Ashford, M. L. (2003) J. Physiol. (Lond.) 547, 417–425
36. Kraft, R., Grimm, C., Grosse, K., Hoffmann, A., Sauerbruch, S., Kettenmann, H., Schultz, G., and Harteneck, C. (2004) Am. J. Physiol. 286, C129–C137
37. Gamaley, I. A., and Klyubin, I. V. (1999) Int. Rev. Cytol. 188, 203–255
38. Berridge, M. J. (1997) Nature 386, 759–760
39. Berridge, M. J., Bootman, M. D., and Lipp, P. (1998) Nature 395, 645–648
40. Sneyd, J., Tzanava-Atanasova, K., Yule, D. I., Thompson, J. L., and Shuttleworth, T. J. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 1392–1396
41. Bootman, M. D., Collins, T. J., Peppiatt, C. M., Prothero, L. S., Mackenzie, L., De Smet, P., Travers, M., Toovey, S. C., Seo, J. T., Berridge, M. J., Ciccolini, F., and Lipp, P. (2001) Semin. Cell Dev. Biol. 12, 3–10
42. Xia, R., Stangler, T., and Abramson, J. J. (2000) J. Biol. Chem. 275, 36556–36561
43. Meissner, G. (2004) Circ. Res. 94, 418–419
44. Missiaen, L., Callewaert, G., De Smedt, H., and Parys, J. B. (2001) Cell Calcium 29, 111–116
45. Peppiatt, C. M., Collins, T. J., Mackenzie, L., Conway, S. J., Holmes, A. B., Bootman, M. D., Berridge, M. J., Seo, J. T., and Roderick, H. L. (2003) Cell Calcium 34, 97–108
46. Falasca, M., Logan, S. K., Lehto, V. P., Baccante, G., Lemmon, M. A., and Schlessinger, J. (1998) EMBO J. 17, 414–422
47. Berridge, M. J., Bootman, M. D., and Roderick, H. L. (2003) Nat. Rev. Mol. Cell Biol. 4, 517–529
48. Bootman, M. D., Taylor, C. W., and Berridge, M. J. (1992) J. Biol. Chem. 267, 25113–25119
49. Vanlingen, S., Sipma, H., De Smet, P., Callewaert, G., Missiaen, L., De Smedt, H., and Parys, J. B. (2001) Biochem. Pharmacol. 61, 803–809
50. Bultrynck, G., Szulczek, K., Kasi, N. N., Assafa, Z., Callewaert, G., Missiaen, L., Parys, J. B., and De Smedt, H. (2004) Biochem. J. 381, 87–96
51. Hu, Q., Yu, Z. X., Ferrans, V. J., Takeda, K., Irani, K., and Ziegelstein, R. C. (2002) J. Biol. Chem. 277, 32546–32551
52. Holbro, T., and Hynes, N. E. (2004) Annu. Rev. Pharmacol. Toxicol. 44, 195–217
53. Rhee, S. G. (1991) Trends Biochem. Sci. 18, 297–301
54. Sundaresan, M., Yu, Z. X., Ferrans, V. J., Sulciner, D. J., Gutkind, J. S., Irani, K., Goldschmidt-Clermont, P. J., and Finkel, T. (1996) Biochem. J. 318, 379–382
55. Carpenter, G. (2000) BioEssays 22, 697–707
56. Peppelenbosch, M. P., Tertoolen, L. G., van der Vlies-Smits, A. M., Qiu, R. G., Mrabet, L., Symons, M. H., de Laat, S. W., and Bos, J. L. (1996) J. Biol. Chem. 271, 7883–7886
57. Rabchevsky, A. G., Weinitz, J. M., Couplier, M., Fages, C., Tinel, M., and Junier, M. P. (1998) J. Neurosci. 18, 10541–10552
58. Levison, S. W., Jiang, F. J., Stoltzfus, O. K., and Ducceschi, M. H. (2000) Glia 32, 328–337