We have investigated possible roles of RhoA and H_{2}O_{2} in the elevation of intracellular Ca^{2+} in Rat-2 fibroblasts. PA induced a transient elevation of [Ca^{2+}]_{i} in the presence or absence of EGTA. Lysophosphatidic acid (LPA) also increased [Ca^{2+}]_{i}, but the sustained Ca^{2+} response was inhibited by EGTA. LPA stimulated the production of inositol phosphates, but PA did not. In the presence of EGTA, preincubation with LPA completely blocked the subsequent elevation of [Ca^{2+}]_{i} by PA, but not vice versa. PA stimulated the translocation of RhoA to the particulate fraction as did LPA. Scrape loading of C3 transferase inhibited the transient Ca^{2+} response to PA, but not to LPA, suggesting an essential role of RhoA in the elevation of [Ca^{2+}]_{i}.PA, H_{2}O_{2} also induced a transient increase of [Ca^{2+}]_{i}, as did PA. H_{2}O_{2} scavengers, catalase and N-acetyl-l-cysteine, completely blocked the rise of [Ca^{2+}]_{i} stimulated by PA, but not by LPA. Furthermore, preincubation with PA blocked the subsequent Ca^{2+} response to H_{2}O_{2}, and the incubation with H_{2}O_{2} also blocked the PA-induced rise of [Ca^{2+}]_{i}. Thus, it was suggested that PA stimulated Ca^{2+} release from PA-sensitive, but not inositol 1,4,5-trisphosphate-sensitive, Ca^{2+} stores by the activation of RhoA and intracellular H_{2}O_{2}.

The hydrolysis of phosphatidylincholines by phospholipase D produces phosphatidic acid (PA) in various cell types stimulated with agonists (1, 2). PA has several physiological functions as a second messenger in cell regulations such as protein phosphorylation (3), expression of c-fos and c-myc (4), stimulation of DNA synthesis (4, 5), activation of stress fiber formation (6–8), and the production of diacylglycerol (2, 9). It is well known that PA is converted into diacylglycerol by the action of PA phosphohydrolase (2, 9), which contributes to the activation of Ca^{2+}-dependent protein kinase C (10). PA is also known to activate phospholipase C-y1 and raf-1 in A431 and MDCK cells, respectively (11, 12). It has also been reported that PA was involved in the activation of superoxide anion production by activating NADPH oxidase (13–15).

Another important role of PA in cell signalings is the increase of intracellular Ca^{2+} ([Ca^{2+}]_{i}) (4). It is known that [Ca^{2+}]_{i} is regulated by the release of Ca^{2+} from intracellular stores and influx from extracellular sources (16). PA has been known to increase [Ca^{2+}]_{i} by the activation of Ca^{2+} influx from internal stores (4, 17), even though there have been reports suggesting the activation of Ca^{2+} influx by PA (18, 19). However, there have been contradictory reports on the mechanisms by which PA triggers Ca^{2+} release from internal stores (4, 17). In A431 cells, PA was shown to elevate [Ca^{2+}]_{i}, by stimulating the hydrolysis of phosphoinositides (4). Contrary to this report, PA could increase [Ca^{2+}]_{i} in the presence of heparin in Jurkat cells, suggesting that PA stimulated Ca^{2+} influx from inositol 1,4,5-trisphosphate (IP_{3})-insensitive stores (17). However, the detailed mechanisms by which PA increases [Ca^{2+}]_{i}, are not still known.

In this report, we present a new possible mechanism of [Ca^{2+}]_{i}, increase in response to PA in Rat-2 fibroblasts. The roles of RhoA in the elevation of intracellular H_{2}O_{2} and [Ca^{2+}]_{i} were studied by scrape loading of C3 transferase into the cells. C3 transferase is known to regulate the activity of RhoA by ADP-ribosylation in several cell functions, including stress fiber formation, focal adhesions, and cell motility (20, 21). The introduction of C3 transferase into the Rat-2 cells inhibited the increase of [Ca^{2+}]_{i}, and intracellular H_{2}O_{2} stimulated by PA. Furthermore, H_{2}O_{2} scavengers, catalase and N-acetylcysteine (NAC), blocked the PA-induced elevation of [Ca^{2+}]_{i}. These observations support the idea that PA increases [Ca^{2+}]_{i}, by activating RhoA and the production of intracellular H_{2}O_{2}. Materials and Methods

**Chemicals and Reagents**—LPA, NAC, EGTA, and catalase from Aspergillus niger were purchased from Sigma. PA (dioleoyl, C18:1, [cis]-9) was obtained from Sigma and further purified by using a solvent system of ethyl acetate/isooctane/acetetic acid/water (130:20:30:100, v/v/v) to remove any possible effect of LPA. Fetal bovine serum, bovine serum albumin, penicillin/streptomycin solution, and Dulbecco’s modified Eagle’s medium were from Life Technologies, Inc. H_{2}O_{2} was obtained from Junsei (Tokyo, Japan). N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) phallacidin and fluo-3/AM were from Molecular Probes (Eugene, OR). Monoclonal anti-RhoA antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemical agents were of analytical grade.

**Cell Culture**—Rat-2 fibroblasts, obtained from American Type Culture Collection (ATCC CRL 1764), were cultured for 2 days at 37 °C in Dulbecco’s modified Eagle’s medium containing 25 mg/mL HEPES, 10% (v/v) fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin (culture medium). Then, the cells were incubated for 2 days at 37 °C with Dulbecco’s modified Eagle’s medium supplemented with 5 μg/mL apotransferrin (Sigma), 1 mg/ml bovine serum albumin, 25 μM HEPES, pH 7.4, 2 mM glutamine, 100 μg/mL streptomycin, and 100 units/ml penicillin (serum-free medium).
Confluent Microscopic Observation of F-actin—F-actin was observed by the procedures of Jung et al. (22). Briefly, Rat-2 cells were grown on round coverslips in a 12-well culture plate and serum-starved for 2 days. After stabilizing in fresh serum-free medium for 1 h, the cells were incubated with 1 μg/ml LPA or 10 μM purified PA for 30 min and fixed with 3.7% (v/v) formaldehyde in Dulbecco’s phosphate-buffered saline (DPBS) (1.2 mM KH2PO4, 8.1 mM Na2HPO4, 0.9 mM CaCl2, 2.7 mM KCl, 0.5 mM MgCl2, and 138 mM NaCl) at pH 7.6 for 30 min. Then, the cells were permeabilized with 0.2% (v/v) Triton X-100 in DPBS for 15 min and stained with 0.165 μM of N-(6-nitrobenz-2-oxa-1,3-diazol-4-yl) phallacidin for 30 min at room temperature. Stained cells were mounted on slide glasses with Gelvatol and observed with a laser scanning confocal microscope (Carl Zeiss LSM 410). Gelvatol was prepared by mixing 100 ml of 0.23% polyvinyl alcohol in DPBS with 50 ml of glycerol. Samples were excited by a 488 nm argon laser, and the images were filtered by a longpass 515 nm filter. Three-dimensional images were constructed from 5–10 serial images (each 1-μm thick) made by automatic optical sectioning.

Scrape Loading of C3 Transferase—C3 transferase, prepared by the procedures of Leem et al. (23), was loaded into Rat-2 cells according to the modified procedures of Malcolm et al. (21). Briefly, Rat-2 cells, grown on a 100-mm culture dish, were scraped in 1 ml of DPBS containing 200 ng of C3 transferase and resuspended in culture medium. Then, cell suspensions were transferred into a six-well culture plate and cultured for 1 day. The cultures were serum-starved by sequential incubation with culture medium containing 0.5% (v/v) fetal bovine serum and serum-free medium each for 1 day and then used for measurements of [Ca2+]i. The incorporation of C3 transferase was checked by immunostaining and co-incorporation of fluorescein isothiocyanate-conjugated dextran.

Measurement of [Ca2+]i—[Ca2+]i was measured by the use of a laser scanning confocal microscope (22). Cells, grown on coverslips and serum-starved for 2 days, were incubated with 4 μM fluo-3,AM in serum-free medium for 40 min and washed three times with serum-free medium. Each coverslip containing stained cells was mounted on a perfusion chamber (self-designed), subjected to a confocal laser scanning microscope (Carl Zeiss LSM 410), and then scanned every second with a 488 nm excitation argon laser and a 515 nm longpass emission filter. Agonists were added to the cells by using an automatic pumping system (self-designed). Sometimes, cells were pretreated with 500 units/ml A. niger catalase for 30 min or 20 μM NAC for 1 h. All images (about 200 images) from the scanning were processed to analyze changes of [Ca2+]i, in a single cell level. The results were expressed as the relative fluorescence intensity. Scrape Loading of RhoA—Cells were incubated with 10 μM PA or 1 μg/ml LPA for 30 min, scraped in DPBS, and harvested by microcentrifugation. The cells were then resuspended in 0.2 ml of lysis buffer (137 mM NaCl, 8.1 mM Na2HPO4, 2.7 mM KCl, 1.5 mM KH2PO4, 2.5 mM EDTA, 1 mM dithiothreitol, 0.1% (w/v) phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, pH 7.5) and disrupted by twenty passes through a 21-gauge needle on ice. The cytosolic fraction was separated from the particulate fraction by centrifugation at 10,000 × g for 1 h and precipitated with 5 volumes of acetone. SDS-polyacrylamide gel electrophoresis was performed using 12% acrylamide, and proteins were transferred onto polyvinylidene difluoride membranes using a Novex wet transfer unit. The membranes were blocked overnight with TBS (DPBS containing 0.01% Tween 20) with 5% (w/v) non-fat dried milk. The blots were incubated for 2 h with anti-RhoA antibody in TBS and further incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h. Then, the blots were developed using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech).

**RESULTS**

**PA Increases [Ca2+]i from PA-sensitive Stores**—In order to study the mechanism by which PA induces the increase in [Ca2+]i, the level of [Ca2+]i, was measured after treating cells with purified PA in Rat-2 fibroblasts. As shown in Fig. 1A, PA caused a rapid, but transient, increase in [Ca2+]i, consistent with previous reports (4, 17). The Ca2+ response was only slightly attenuated when external Ca2+ was eliminated by treating with EGTA, suggesting that PA increased [Ca2+]i by triggering Ca2+ efflux from internal stores rather than stimulating Ca2+ influx from extracellular source. LPA also increased [Ca2+]i, but EGTA inhibited sustained Ca2+ response to LPA, indicating that LPA elevated [Ca2+]i by triggering both Ca2+ efflux from internal stores and the influx of extracellular Ca2+ (Fig. 1B). In contrast, EGTA completely blocked the increase of cytosolic Ca2+ produced by arachidonic acid (Fig. 1C). Since there have been inconsistent reports on the involvement of IPs in PA-induced increase of [Ca2+]i (4, 17), the amount of IPs was measured after treating Rat-2 cells with purified PA in the presence of 20 mM LiCl (Fig. 2). The hydrolysis of phosphoinositides by phospholipase C is known to increase inositol phosphates in the presence of LiCl (16, 25). As expected from the previous reports (22, 26–28), incubation with LPA increased IPs by about 4-fold over the control level. However, PA did not cause any changes in the amount of IPs. So PA expected to mediate IP3 formation in PA-induced [Ca2+]i response (4, 17).

**FIG. 1. Changes of [Ca2+]i by PA (A), LPA (B), or arachidonic acid (C).** Serum-starved Rat-2 cells were labeled with 4 μM fluo-3-AM for 40 min and treated with 10 μM PA, 1 μg/ml LPA, or 100 μM arachidonic acid for the indicated times, in the presence (EGTA−) or presence (EGTA+) of 4 mM EGTA. Then, [Ca2+]i was monitored using a laser scanning confocal microscope as explained under "Materials and Methods." Results are expressed as the relative fluorescence intensity (RFI). Each trace is a single cell representative from at least four separate experiments.
RhoA was also involved in the PA-induced increase of 

Ca$^{2+}$ by C3 transferase (8), we have investigated the possibility that PA stimulated stress fiber formation, which was inhibited by the incubation with LPA (data not shown). So if LPA triggered Ca$^{2+}$ efflux from PA-sensitive pool, the preincubation with LPA should block the elevation of [Ca$^{2+}$], induced by PA. As shown in Fig. 3A, the preincubation with LPA, in the presence of EGTA, completely blocked the increase of [Ca$^{2+}$] stimulated by PA. However, the depletion of PA-sensitive Ca$^{2+}$ pool by the incubation with PA showed a small inhibitory effect on the subsequent Ca$^{2+}$ response to LPA (Fig. 3B). Thus, these results strongly suggested that LPA increased [Ca$^{2+}$], from IP$_3$-sensitive pools and also from PA-sensitive (IP$_3$-independent) Ca$^{2+}$ pool.

PA Increases [Ca$^{2+}$], by RhoA—Since it has been reported that PA stimulated stress fiber formation, which was inhibited by C3 transferase (8), we have investigated the possibility that RhoA was also involved in the PA-induced increase of [Ca$^{2+}$. First, we have tested possible activation of RhoA by PA by measuring the translocation of RhoA, since it is known that RhoA is redistributed from the cytosolic to the particulate fraction after being activated (21). As shown in Fig. 4, most of RhoA was localized in the cytosolic fraction in unstimulated cells, and a fraction of RhoA was translocated to the membrane fraction in response to PA. As expected from a previous report (21), LPA also activated the translocation of RhoA to the membrane fraction. Next, the role of RhoA in the PA-induced increase of [Ca$^{2+}$] was investigated by scrape loading of C3 transferase into Rat-2 cells. It is known that C3 transferase inhibited the activity of RhoA by ADP-ribosylation at Asp$^{41}$ (20). The effect of incorporated C3 transferase on RhoA was shown by the inhibition of stress fiber formation in response to PA (Fig. 5A). In the control cells, scraped without C3 transferase, PA stimulated stress fiber formation, which was completely inhibited in C3 transferase-loaded cells. C3 transferase also blocked the stress fiber formation induced by LPA (data not shown). The effect of C3 transferase on the elevation of [Ca$^{2+}$], by PA was shown in Fig. 5B. C3 transferase inhibited the increase of [Ca$^{2+}$], stimulated by PA, suggesting an essential role of RhoA in the PA-stimulated increase of [Ca$^{2+}$]. However, the toxin had no significant effect on the elevation of [Ca$^{2+}$], in response to LPA, possibly because LPA could release Ca$^{2+}$ from IP$_3$-sensitive pool, even though PA-sensitive Ca$^{2+}$ pool was blocked. Taken together, it was suggested that RhoA played an essential role in the PA-induced increase of [Ca$^{2+}$].

The Role of H$_2$O$_2$ in the PA-induced Ca$^{2+}$ Increase—it has been reported that H$_2$O$_2$ stimulates the increase of [Ca$^{2+}$], in various cells (29–31). Recently, we have observed that PA produced intracellular H$_2$O$_2$ by over 6-fold and the increase was blocked by C3 transferase in Rat-2 cells. So we have investigated any possible role of H$_2$O$_2$ in the PA-induced rise of [Ca$^{2+}$]. First, we measured the changes of [Ca$^{2+}$], after treating the cells with exogenous H$_2$O$_2$ (Fig. 6A). H$_2$O$_2$ induced a transient elevation of [Ca$^{2+}$], as did PA, consistent with previous reports (30, 31). The transient response of [Ca$^{2+}$], to H$_2$O$_2$ was not largely affected by the pretreatment with EGTA, indicating that H$_2$O$_2$ increased [Ca$^{2+}$], from the internal stores as did PA (Fig. 1). To confirm the role of H$_2$O$_2$ in the regulation of [Ca$^{2+}$], the cells were preincubated with H$_2$O$_2$ scavengers, catalase and NAC, and then the changes of [Ca$^{2+}$], by PA were investigated (Fig. 6B). Catalase completely blocked the increase of [Ca$^{2+}$], stimulated by PA, but had no effect on the subsequent increase by LPA (Fig. 6B). NAC also blocked the Ca$^{2+}$ response to PA, but not to LPA (Fig. 6C). These results suggested that intracellular H$_2$O$_2$ may play a role in the PA-induced increase of [Ca$^{2+}$].

The role of H$_2$O$_2$ was further confirmed by serial incubations of

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Rat-2 cells with H2O2 and PA in the presence of EGTA (Fig. 7). The incubation with PA induced a transient increase of [Ca2+]i, but the subsequent incubation with H2O2 did not cause any changes in [Ca2+]i (Fig. 7A). Preincubation with H2O2 also blocked the Ca2+ response to PA (Fig. 7B), suggesting that PA and H2O2 caused Ca2+ release from the same stores. However, the depletion of H2O2-sensitive or PA-sensitive Ca2+ stores did not block the subsequent response of [Ca2+]i to LPA (Figs. 3B and 7C). Thus, it was concluded that H2O2 increased [Ca2+]i by triggering Ca2+ efflux from PA-sensitive, but not from IP3-sensitive stores.

**DISCUSSION**

In the present report we have shown that PA increased [Ca2+]i from PA-sensitive internal stores, and the transient Ca2+ response was dependent on RhoA and H2O2. PA induced a transient increase of [Ca2+]i in the absence of extracellular Ca2+, whereas the sustained Ca2+ increase by LPA was dependent on the extracellular Ca2+. PA stimulated the translocation of RhoA to the membrane fraction, and C3 transferase inhibited the PA-induced elevation of [Ca2+]i. H2O2 scavengers, catalase and NAC, also blocked the rise of [Ca2+]i by PA. Furthermore, the depletion of PA-sensitive Ca2+ stores inhibited the Ca2+ response to H2O2, and vice versa.

Now, it is likely that PA stimulates Ca2+ release from PA-sensitive stores, which is not dependent on IP3. In Jurkat T cells, the depletion of IP3-sensitive Ca2+ stores by incubation with an anti-CD3 antibody OKT3 (an IP3-generating drug), in the presence of EGTA, did not affect on the elevation of [Ca2+]i by the subsequent incubation of PA (17). In the same cells, heparin inhibited the rise in [Ca2+]i by IP3, but did not by PA, suggesting that PA increased [Ca2+]i, from IP3-insensitive stores. It has been also reported that sphingosine 1-phosphate could produce PA by activating phospholipase D and also increased [Ca2+]i from internal stores by an IP3-independent mechanism in Swiss 3T3 cells (25, 32). Consistent with the previous reports, our results showed that PA produced a transient increase of [Ca2+]i from PA-sensitive stores without the production of IP3 (Figs. 1 and 2).

To our understanding, this is the first report that the rise of [Ca2+]i by PA was dependent on RhoA. PA activated the translocation of RhoA to the membrane fraction, and C3 transferase inhibited the transient Ca2+ response to PA (Fig. 4). It has been reported that C3 transferase induced in vivo ADP-ribosylation in a dose-dependent manner in Rat-2 cells (23). Previously, there have been reports indicating possible roles of RhoA in the regulation of [Ca2+]i, and smooth muscle contraction (33–35). In C3H 10/12 cells, microinjection of C3 transferase inhibited the increase of [Ca2+]i, in response to thrombin and platelet-derived growth factor (33). Ca2+ waves induced by IP3 were retarded by C3 transferase in Xenopus oocytes (34). It has been also reported that RhoA increased Ca2+ sensitivity of arterial smooth muscle contraction (35). Thus, it is likely that RhoA plays an essential role in the regulation of [Ca2+]i.

H2O2 is known to increase [Ca2+]i in various cells, including...
smooth muscle cells, macrophages, and endothelial cells (29–31). In rat alveolar macrophages, H$_2$O$_2$ stimulated the release of Ca$^{2+}$ from IP$_3$-independent stores, which was essential for respiratory burst (30). The role of H$_2$O$_2$ in the elevation of [Ca$^{2+}$]$_i$ was also shown in endothelial cells (31). Superoxide and H$_2$O$_2$ produced by the incubation with xanthine oxidase and xanthine at nontoxic doses induced a transient rise of [Ca$^{2+}$]$_i$, but the Ca$^{2+}$ response was mainly induced by H$_2$O$_2$ rather than superoxide in these cells (31). Our results also supported the previous reports. H$_2$O$_2$ caused a transient Ca$^{2+}$ increase in the presence or absence of extracellular Ca$^{2+}$. H$_2$O$_2$ scavengers, NAC and catalase, completely inhibited the rise of [Ca$^{2+}$]$_i$ by PA. Furthermore, we have observed that PA increased the amount of intracellular H$_2$O$_2$ and the increase was blocked by C3 transferase. Considering an essential role of RhoA in the PA-stimulated increase of [Ca$^{2+}$]$_i$, it was concluded that PA increased [Ca$^{2+}$]$_i$ by the activation of RhoA and the subsequent production of intracellular H$_2$O$_2$.

Interestingly, our results suggested that LPA triggered Ca$^{2+}$ release from PA-sensitive stores. We have previously observed that LPA produced PA by stimulating phospholipase D in Rat-2 cells (22). The depletion of PA-sensitive Ca$^{2+}$ stores did not block the subsequent Ca$^{2+}$ increase by LPA, but preincubation with LPA blocked the Ca$^{2+}$ response to PA (Fig. 3), indicating that LPA triggered Ca$^{2+}$ efflux from PA-sensitive stores. C3 transferase, which inhibited the elevation of [Ca$^{2+}$]$_i$ by PA, had no inhibitory effect on the Ca$^{2+}$ response to LPA (Fig. 5). In addition, H$_2$O$_2$ scavengers, catalase and NAC, inhibited the Ca$^{2+}$ elevation by PA, but not by LPA (Fig. 6). PA activated the translocation of RhoA to the membrane fraction and the production of H$_2$O$_2$. The differential effects of C3 transferase and H$_2$O$_2$ scavengers on the Ca$^{2+}$ response to PA and LPA were explained by the production of IPs by LPA (Fig. 2). Thus, it is likely that LPA increases [Ca$^{2+}$], from both PA- and IP$_3$-sensitive stores.

What is the possible role of the intracellular H$_2$O$_2$ and Ca$^{2+}$ increased by PA? One possibility is to play a role in the actin polymerization stimulated by PA. It has been reported that Ca$^{2+}$ may be involved in the actin polymerization by regulating the interaction of actin with Ca$^{2+}$-dependent actin-binding proteins such as gelsolin and villin (36). In Rat-2 cells, PA stimulated RhoA and the elevation of intracellular H$_2$O$_2$ and [Ca$^{2+}$]$_i$. C3 transferase inhibited the production of H$_2$O$_2$ and stress fiber formation (Fig. 5) activated by PA. Catalase also inhibited PA-induced stress fiber formation (data not shown). H$_2$O$_2$ increased [Ca$^{2+}$]$_i$ (Figs. 6 and 7) and stimulated stress fiber formation as did PA (data not shown). So it was suggested that PA activated stress fiber formation by a sequential activation of RhoA and the increase of intracellular H$_2$O$_2$ and [Ca$^{2+}$]$_i$. However, H$_2$O$_2$ alone produced fragmented F-actins in the Rat-2 cells loaded with C3 transferase, indicating that stress fiber formation may require additional kinases activated by RhoA, such as Rho kinase and protein kinase N (37–39). So, RhoA may regulate PA-induced stress fiber formation by activating its downstream components, H$_2$O$_2$/Ca$^{2+}$ and RhoA-activated kinases, even though it is necessary to elucidate their roles in the actin polymerization activated by PA.

### REFERENCES

Ca$^{2+}$ Increase Mediated by RhoA and $H_2O_2$

Phosphatidic Acid-induced Elevation of Intracellular Ca\textsuperscript{2+} Is Mediated by RhoA and H\textsubscript{2}O\textsubscript{2} in Rat-2 Fibroblasts
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doi: 10.1074/jbc.273.21.12710

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