Amplification of Ca$^{2+}$ Signaling by Diacylglycerol-mediated Inositol 1,4,5-Trisphosphate Production*§

Chihiro Hisatsune‡‡, Kyoko Nakamura†, Yukiko Kuroda‡, Takeshi Nakamura†, and Katsuhiko Mikoshiba‡‡

From the ‡‡Laboratory for Developmental Neurobiology, RIKEN Brain Science Institute (BSI) 2-1 Hirosawa, Wako City, Saitama 351-0198, Japan, ‡Calcium Oscillation Project, JICORP, Japan Science and Technology Agency, 3 Nibancho, Chiyoda-ku, Tokyo 102-0084, Japan, and ‡Division of Molecular Neurobiology and ‡‡Division of Neural Signal Information NTT-IMSUT, Institute of Medical Science, University of Tokyo, 3-4-1, Shirokane-dai, Minato-ku, Tokyo 108-8639, Japan

Received for publication, August 19, 2004, and in revised form, December 10, 2004
Published, JBC Papers in Press, January 6, 2005, DOI 10.1074/jbc.M409535200

Stimulation of various cell surface receptors leads to the production of inositol 1,4,5-trisphosphate (IP$_3$) and diacylglycerol (DAG) through phospholipase C (PLC) activation, and the IP$_3$ and DAG in turn trigger Ca$^{2+}$ release through IP$_3$ receptors and protein kinase C activation, respectively. The amount of IP$_3$ produced is particularly critical to determining the spatio-temporally coordinated Ca$^{2+}$-signaling patterns. In this paper, we report a novel signal cross-talk between DAG and the IP$_3$-mediated Ca$^{2+}$-signaling pathway. We found that a DAG derivative, 1-oleoyl-2-acetyl-sn-glycerol (OAG), induces Ca$^{2+}$ oscillation in various types of cells independently of protein kinase C activity and extracellular Ca$^{2+}$. The OAG-induced Ca$^{2+}$ oscillation was completely abolished by depletion of Ca$^{2+}$ stores or inhibition of PLC and IP$_3$ receptors, indicating that OAG stimulates IP$_3$ production through PLC activation and thereby induces IP$_3$-induced Ca$^{2+}$ release. Furthermore, intracellular accumulation of endogenous DAG by a DAG-lipase inhibitor greatly increased the number of cells responding to agonist stimulation at low doses. These results suggest a novel physiological function of DAG, i.e. amplification of Ca$^{2+}$ signaling by enhancing IP$_3$ production via its positive feedback effect on PLC activity.

Stimulation of a wide variety of cells by hormones, neurotransmitters, or growth factors leads to the activation of phospholipase C (PLC)$^1$ and triggers inositol 1,4,5-trisphosphate (IP$_3$)-mediated Ca$^{2+}$ signaling via activation of IP$_3$ receptors (IP$_3$Rs) on the endoplasmic reticulum (1, 2). Proper regulation of receptor-IP$_3$/Ca$^{2+}$ signaling is very important, because the IP$_3$-induced Ca$^{2+}$ release underlying a variety of spatio-temporal Ca$^{2+}$ dynamics has been shown to considerably affect various cellular functions such as smooth muscle contraction, fertilization, immune response, gene expression, synaptic plasticity, development, and so on (3, 4). The molecular mechanisms responsible for the versatility of IP$_3$-mediated Ca$^{2+}$ signaling include (i) a plasma membrane receptor-PLC system that generates the second messenger IP$_3$ (5–7), (ii) IP$_3$Rs (8, 9), (iii) Ca$^{2+}$ sequestration mechanisms (10, 11), (iv) IP$_3$ phosphatases and kinases (12, 13), and (v) Ca$^{2+}$ influx machinery (14). These molecular mechanisms interact with each other, and thus, they all affect each other. For example, intracellular Ca$^{2+}$ released from Ca$^{2+}$ stores has a positive feedback effect on PLC activity (15, 16). IP$_3$R activity is positively or negatively regulated by Ca$^{2+}$ depending on the intracellular Ca$^{2+}$ level (17), and Ca$^{2+}$ entry machinery is regulated by store depletion, conformational changes of IP$_3$Rs, or direct binding of phospholipids and DAG (14). Thus, numerous regulatory mechanisms of these signaling molecules and their interactions allow cells to establish precise and complex Ca$^{2+}$ patterns that contribute to various physiological phenomena.

In this paper, we report a novel regulatory mechanism involved in receptor-IP$_3$-Ca$^{2+}$ signaling, i.e. DAG-mediated positive feedback stimulation of PLC. We unexpectedly found that 1-oleoyl-2-acetyl-sn-glycerol (OAG), a membrane-permeable derivative of DAG, induces Ca$^{2+}$ oscillation in COS-7 cells independently of PKC activity and extracellular Ca$^{2+}$. The OAG-induced Ca$^{2+}$ oscillation was also observed in HeLa cells, CHO-K1 cells, and astrocytes, suggesting that the OAG-induced Ca$^{2+}$ oscillation is a general phenomenon in various types of cells. The OAG-induced Ca$^{2+}$ oscillation was dependent on Ca$^{2+}$ release through IP$_3$Rs, because 2-aminoethyl diphenylborinate (2-APB) (18), an IP$_3$R inhibitor, or depletion of Ca$^{2+}$ stores with cyclopiazonic acid (CPA) abolished the Ca$^{2+}$ mobilization. In addition, we found that treatment of COS-7 cells with a PLC inhibitor or expression of the IP$_3$-absorbent protein “IP$_3$-sponge” completely abolished the OAG-induced Ca$^{2+}$ oscillation, indicating that OAG stimulates IP$_3$ production via PLC activation. We also discovered that accumulation of endogenous DAG as a result of exposure to a DAG-lipase inhibitor increases the sensitivity of the Ca$^{2+}$ response in COS-7 cells to low-dose ATP stimulation. These findings suggested that DAG produced via the receptor-PLC signaling cascade leads to further PLC activation, resulting in the increased IP$_3$ production and amplification of receptor-Ca$^{2+}$ signaling. We propose that, in addition to the well known positive feedback effect of Ca$^{2+}$ on PLC activity, this novel signal cross-talk...
between DAG and PLC activity may be a crucial mechanism regulating the amount of IP$_3$, which is an important factor in determining the threshold of Ca$^{2+}$ signaling generation and Ca$^{2+}$ dynamics in response to agonist stimulation, and that it may play an important role in physiological phenomena that are dependent on IP$_3$-induced Ca$^{2+}$ release.

**EXPERIMENTAL PROCEDURES**

**Culture**—COS-7 cells, CHO-K1, and HeLa cells were cultured in Dulbecco's modified essential medium (DMEM) (Nakarai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 µg/ml streptomycin (Nakarai). Astrocytes were prepared from the cerebral cortex of postnatal 1 day mice or postnatal 1 day Wistar rats using the standard method (19). Following trypsin treatment, cortices were dissociated by trituration and cultured in 75-mm$^2$ flask with DMEM containing 10% fetal bovine serum, 50 units/ml penicillin, and 50 µg/ml streptomycin. The confluent cells then were treated with trypsin after overnight shaking (280 rpm) and replated on 3.5 cm of poly-l-lysine-coated glass-bottom dishes (Matsunami, Osaka, Japan) at a density of 1 × 10$^5$/dish. After 4–5 days from the replating, Ca$^{2+}$ imaging was performed. We confirmed that the cultured cells were astrocytes by immunostaining with mouse anti-glial fibrillary acidic protein antibody (Sigma).

**Pharmacological Reagents**—The following reagents were used in study: 1-octanol-2-acetyl-sn-glycerol (OAG) (Calbiochem, Nakarai Tesque, and Avanti Polar Lipids, Alabaster, AL). To notice the possible effect caused by contaminated substances, we used OAG purchased from the three companies and confirmed their same effect on Ca$^{2+}$ mobilization: TAME IP$_3$ (TPA) (Calbiochem); 1-earosor-2-archichidonyl-sn-glycerol (SAG) (Sigma); U73122 and U73343 (Calbiochem); RH5802967 (Calbiochem); 2-APB; staurosporine (Calbiochem); phorbol 12-myristate 13-acetate (PMA) (Calbiochem); CPA (Calbiochem); inositol 1,4,5-triphosphate (Djindo, Kumamoto, Japan); and ATP.

**GFP and IP$_3$ Sponge Transfection**—COS-7 cells plated on the 3.5-cm poly-l-lysine-coated glass bottom dish (Matsunami) were transfected with totally 1.0 µg of plasmids encoding GFP (pcDNA-N3 (Clontech, Palo Alto, CA)) and IP$_3$-sponge (pcDNA-IP3-sponge). The G224 fragment of fos gene with totally 1.0 µg of plasmids encoding GFP (pcDNA-N3 (Clontech, Palo Alto, CA)) and IP$_3$-sponge (pcDNA-IP3-sponge) was cloned into pcDNA3 (Clontech). The GFP- and IP$_3$-sponge encoding IP3-sponge (20) was cloned into pcDNA3 (Clontech) using this solution. DAG kinase assay was performed as reported previously (22, 23). After separating DAG using thin-layer chromatography, the thin-layer chromatography plate was subjected to autoradiography and the optical density of radioactive spot corresponding to DAG was quantified using a custom-made software for image analysis (TI-workbench).

**RESULTS**

A DAG Derivative, OAG, Induces Ca$^{2+}$ Oscillation in Various Types of Cells—To explore a novel biological function of DAG in intracellular signaling, we measured Ca$^{2+}$ signals following the application of a membrane-permeable DAG derivative, OAG, to COS-7 cells. Interestingly, we found that, in the nominal absence of external Ca$^{2+}$, OAG application (100 µM) to COS-7 cells induces Ca$^{2+}$ oscillation with a short latency of 1–5 min (Fig. 1A; also see Supplemental Video 1). The frequency of Ca$^{2+}$ oscillation varies from cell to cell. Although the nominal Ca$^{2+}$-free solution (EGTA-free) actually contains very low concentration of Ca$^{2+}$ (~1.0 µM), the Ca$^{2+}$ mobilization following OAG application could not have been due to Ca$^{2+}$ influx through cell surface Ca$^{2+}$-permeable channels such as TRPC channels as previously reported (24–26) because we also detected the OAG-induced Ca$^{2+}$ mobilization in the extracellular solution containing 0.5 mM EGTA (Fig. 1B). The application of a membrane-impermeable DAG derivative, SAG (100 µM), failed to induce Ca$^{2+}$ oscillation (Fig. 1C). The effect of OAG was dose-dependent. As shown in Fig. 1D, hardly any Ca$^{2+}$ mobilization was detectable when 10 µM OAG was applied. The OAG-induced Ca$^{2+}$ oscillation was first observed at 30 µM OAG, and the frequency of Ca$^{2+}$ oscillation gradually increased as the OAG concentration was increased to 30, 100, and 300 µM (Fig. 1D). The time lag between OAG application and the onset of the Ca$^{2+}$ response also tended to become shorter as the OAG concentration was increased. The corresponding concentrations of the vehicle, Me$_2$SO, did not induce any Ca$^{2+}$ oscillations (data not shown). These results were unexpected, because several previous studies have reported detecting no Ca$^{2+}$ mobilization upon OAG application under extracellular-Ca$^{2+}$-free conditions (26–28). Because we detected similar Ca$^{2+}$ oscillations after exposure to OAG purchased from three different companies (see ”Experimental Procedures”), the OAG-induced Ca$^{2+}$ response is unlikely to have been an artifact.

To determine whether OAG would induce Ca$^{2+}$ oscillation in other types of cells, we applied OAG to CHO-K1 cells, HeLa cells, and rat primary astrocytes and examined them for a Ca$^{2+}$ response. As shown in Fig. 2, in the nominal absence of external Ca$^{2+}$, we observed OAG-induced Ca$^{2+}$ mobilization in these cells, the same as in COS-7 cells, suggesting that the OAG-induced Ca$^{2+}$ oscillation is a general phenomenon rather than specific to COS-7 cells.

**Ca$^{2+}$ Release through IP$_3$ Rs Underlies OAG-induced Ca$^{2+}$ Oscillation**—We next evaluated the contribution of Ca$^{2+}$ release from Ca$^{2+}$ stores to the OAG-induced Ca$^{2+}$ oscillation in COS-7 cells. When the Ca$^{2+}$ store had been depleted with a Ca$^{2+}$ pump inhibitor, CPA (10 µM), before OAG application, OAG no longer induced Ca$^{2+}$ oscillation (Fig. 3A). Moreover, the application of 2-APB, an inhibitor of IP$_3$ Rs, completely abolished the OAG-induced Ca$^{2+}$ oscillation (Fig. 3B), suggesting that the OAG-induced Ca$^{2+}$ oscillation was caused by Ca$^{2+}$ release through IP$_3$ Rs. To determine whether OAG activates IP$_3$ Rs directly, we next performed an in vitro Ca$^{2+}$ release assay using mouse cerebellar microsomes. As shown in Fig. 3C, no Ca$^{2+}$ release from cerebellar microsomes was detected in response to 100 µM OAG or the solvent, Me$_2$SO, whereas the application of IP$_3$ (100 µM) to cerebellar microsomes induced transient Ca$^{2+}$ release. In addition, OAG did not enhance the amount of Ca$^{2+}$ release induced by IP$_3$ (Fig. 3C). Therefore, the OAG-induced Ca$^{2+}$ oscillation is likely to be mediated by IP$_3$ production rather than by direct activation of IP$_3$ Rs.
To confirm the possible involvement of IP$_3$ production in the OAG-induced Ca$^{2+}$ oscillation, we exposed the cells to a PLC inhibitor, U73122, prior to OAG application. As shown in Fig. 4, 10 μM U73122 completely abolished the OAG-induced Ca$^{2+}$ oscillation in COS-7 cells (Fig. 4Aa), whereas the biologically inactive analog U73343 (10 μM) had no inhibitory effect (Fig. 4Ab). Furthermore, when we transiently co-expressed IP$_3$-sponge (20) and GFP in COS-7 cells before OAG application, no Ca$^{2+}$ oscillation was detected in GFP-positive IP$_3$-sponge-expressing COS-7 cells, whereas control cells that did not express IP$_3$-sponge (GFP-negative cells) showed Ca$^{2+}$ oscillation (Fig. 4B). Expression of GFP alone did not affect the OAG-induced Ca$^{2+}$ oscillation (data not shown).

Thus, these results strongly suggested that OAG and thus also DAG are capable of inducing production of IP$_3$ via PLC activation, which initiates Ca$^{2+}$ release through IP$_3$Rs.

Analysis of the Molecular Mechanism of the OAG-induced Ca$^{2+}$ Oscillation—To gain further insight into the molecular mechanisms linking DAG to PLC activation, we analyzed the involvement of PKC activity in the OAG-induced Ca$^{2+}$ oscillation. As shown in Fig. 5, the OAG-induced Ca$^{2+}$ oscillation occurred independently of PKC activation, because acute application of PMA, a potent activator of PKC (1.0 μM), to COS-7 cells did not induce any Ca$^{2+}$ oscillation (Fig. 5A) and pretreatment of cells with a broad serine-threonine kinase inhibitor, staurosporine (100 nM), did not abolish the Ca$^{2+}$ mobilization following OAG application (Fig. 5B). Next, because cellular DAG is mainly metabolized by DAG lipase, we treated COS-7 cells with the DAG-lipase inhibitor RHC80267 (25 μM) before OAG application. As shown in Fig. 5C, RHC80267 did not affect the OAG-induced oscillation, indicating that it was not caused by metabolites of OAG. Interestingly, we found that a Src family tyrosine kinase inhibitor, PP2, completely abolished the OAG-induced Ca$^{2+}$ oscillation, whereas the inactive PP2 analog PP3 used as a control failed to abolish the OAG-induced Ca$^{2+}$ oscillation (Fig. 5D).

Endogenous DAG Modifies the Ca$^{2+}$ Response to ATP Stimulation in COS-7 Cells—Finally, we tried evaluating the contribution to the intracellular Ca$^{2+}$ mobilization in COS-7 cells of DAG endogenously produced in response to ATP stimulation. We hypothesized that if metabolism of endogenously produced DAG was inhibited by DAG-lipase inhibitor, accumulated DAG would cause further production of IP$_3$, resulting in the change in Ca$^{2+}$ mobilization in response to agonist stimulation. The use of the DAG-lipase inhibitor, RHC80267, has been a common method to examine the effect of endogenous DAG on several physiological phenomena (24, 29), and we could detect the increased endogenous DAG level by RHC80267 treatment upon ATP stimulation using DAG kinase assay (Supplemental Fig. 1). To rule out the possible effect of DAG-dependent PKC activity on Ca$^{2+}$ mobilization, we pretreated COS-7 cells with 300 nM PMA for 36 h to down-regulate PKC activity (30) and imaged Ca$^{2+}$ signals evoked by application of various concentrations of ATP (0.05, 0.1, 0.3, and 0.5 μM) in the presence and absence of DAG-lipase inhibitor RHC80267 (50 μM). As shown in Fig. 6, the results showed that, whereas none of the control cells released Ca$^{2+}$ in response to 0.5 μM ATP stimulation (0%), a small population of RHC80267-treated cells did release Ca$^{2+}$ from Ca$^{2+}$ stores in response to 0.05 μM ATP stimulation (0.54 ± 0.59%, mean ± S.D.). In addition, the numbers of RHC80267-treated cells that responded to 0.1, 0.3, and 0.5 μM ATP stimulation were statistically higher than the numbers of control cells (0.1 μM ATP: control 0.15 ± 0.39%; RHC80267 4.51 ± 4.72%; 0.3 μM ATP: control 19.35 ± 14.23%; RHC80267 44.34 ± 15.39%; 0.5 μM ATP: control 58.84 ± 12.47%; RHC80267 70.59 ± 10.79%, mean ± S.D.). Although RHC80267-treated cells tended to have larger Ca$^{2+}$ peak amplitudes than the control cells, there was no statistically sig-
significant difference in peak Ca\(^{2+}\) amplitudes between the RHC80267-treated cells and the control cells. When stimulated with a higher dose of ATP (3.0 \(\mu\)M), almost all of the control and RHC80267-treated cells released Ca\(^{2+}\) and there were no differences between them in the percentage of cells that responded or the amplitudes of the Ca\(^{2+}\) release (data not shown). Thus, these results suggested that a higher level of DAG endogenously produced in response to ATP stimulation could cause further IP\(_3\) production via PLC activation, resulting in the enhanced Ca\(^{2+}\) mobilization from Ca\(^{2+}\) stores through IP\(_3\)-Rs in response to low-dose agonist stimulation.

**DISCUSSION**

DAG is well known as a potent PKC activator; however, other physiological actions of DAG have recently been reported including activation of RasGRP (31) and some TRPC channels (24, 25) and inhibition of cyclic nucleotide-gated channels (32, 33). We propose a new biological function of DAG, i.e. DAG-induced IP\(_3\) production via PLC activation. The results of the present study demonstrated that a DAG derivative, OAG, induces Ca\(^{2+}\) oscillation in various types of cells, and based on the following findings, we concluded that the OAG-induced Ca\(^{2+}\) oscillation was attributable to IP\(_3\)-mediated Ca\(^{2+}\) release through IP\(_3\)-Rs as follows: 1) the independence of extracellular Ca\(^{2+}\); 2) abolition of the oscillation by store depletion; 3) blockade by IP\(_3\)-mediated signaling inhibitors, 2-APB and IP\(_3\)-sponge; and 4) blockade by PLC inhibition. These findings indicate the existence of a positive feedback effect of DAG on PLC activity, and consistent with this finding, we have demonstrated that the DAG-mediated positive feedback signaling...
to PLC contributes to the amplification of agonist-induced Ca\textsuperscript{2+} signaling, suggesting a physiological significance of the feed-
back mechanism.

The amount of IP\textsubscript{3} produced by OAG could be inferred from
the pattern of Ca\textsuperscript{2+} signaling, because the pattern is generally
dependent on the concentration of the agonists applied to cells.
In many types of cells, application of low concentrations of
agonists, which result in the production of small amounts of
IP\textsubscript{3}, induces a transient increase in cytosolic Ca\textsuperscript{2+}—lasting only
a few seconds (Ca\textsuperscript{2+} spike) or repetitive Ca\textsuperscript{2+} spikes (Ca\textsuperscript{2+}
oscillation), whereas high concentrations of agonists induce
sustained Ca\textsuperscript{2+} elevation throughout the period of agonist
application. Because at the highest concentration we tested (300
\mu M OAG (first arrow in right panel), or the solvent 0.1\% Me\textsubscript{2}SO
(DMSO) (first arrow in the left panel) and 100 \mu M IP\textsubscript{3}, second arrow) were applied to
the cerebellar microsomes. Data shown are representative of 5–6 trials. R, ratio.

DAG-mediated Positive Feedback Stimulation of PLC

Fig. 3. OAG-induced Ca\textsuperscript{2+} oscillation in COS-7 cells depends on intracellular Ca\textsuperscript{2+} stores and IP\textsubscript{3R} channel
activity. A, depletion of Ca\textsuperscript{2+} stores by CPA (10 \mu M) inhibited OAG-induced Ca\textsuperscript{2+}
oscillation. The first and second arrows indicate CPA and OAG (100 \mu M) application,
respectively. B, an IP\textsubscript{3R} inhibitor, 2-APB (75 \mu M), blocked the OAG-induced
Ca\textsuperscript{2+} oscillation. C, OAG did not directly stimulate Ca\textsuperscript{2+} release from cerebellar
microsomes, whereas control IP\textsubscript{3} did. After loading Ca\textsuperscript{2+} into the cerebellar micro-
somes via the Ca\textsuperscript{2+}\textsuperscript{+} pump using 1\textsuperscript{st} ATP, 100 \mu M OAG (first arrow in right
panel), or the solvent 0.1\% Me\textsubscript{2}SO (DMSO) (first arrow in the left panel) and
100 \mu M IP\textsubscript{3}, (second arrow) were applied to
the cerebellar microsomes. Data shown are representative of 5–6 trials. R, ratio.

Ca\textsuperscript{2+} entry through TRPC3 channels and that Ca\textsuperscript{2+} release
from internal stores was not involved in the Ca\textsuperscript{2+} oscillation. In
the presence of external Ca\textsuperscript{2+}, we observed similar slow Ca\textsuperscript{2+}
oscillations in glial cells that were less frequent than in the
absence of external Ca\textsuperscript{2+} (data not shown); however, the
percentage of the glial cells exhibiting low-frequency Ca\textsuperscript{2+} oscilla-
tion in response to OAG in the presence of extracellular Ca\textsuperscript{2+}
was even smaller than reported by Grimaldi et al. (28). In
addition, they did not detect the OAG-induced Ca\textsuperscript{2+} mobiliza-
tion observed in the present study in the absence of external
Ca\textsuperscript{2+}. Although we do not know the exact reasons for these
discrepancies, differences among groups in cell culture proto-
cols and conditions and thus in stages of the differentiation of
the glial cells may have been responsible.

Although we found that DAG stimulates PLC activity, it was
previously shown that PKC, which is activated by DAG, inhib-
its PLC activity via phosphorylation of PLC (34). These two
effects of DAG on PLC activity seem inconsistent. Because PKC
phosphorylates and inhibits only the PLC\textsubscript{b} isoform (34), DAG
may stimulate other isoforms of PLC and enhance IP\textsubscript{3}-medi-
ated Ca\textsuperscript{2+} signaling. Alternatively, the above two actions of
DAG may affect different phases of the Ca\textsuperscript{2+} signaling induced
by IP\textsubscript{3}-mobilizing agonists. Because PKC activation requires
cytosolic Ca\textsuperscript{2+} elevation, PLC activity by PKC-mediated phos-
phorylation may become suppressed after cytosolic Ca\textsuperscript{2+}
is sufficiently increased and the suppression may then facilitate
the termination of individual Ca\textsuperscript{2+} spikes and waves. By contrast,
DAG together with the Ca\textsuperscript{2+} released from Ca\textsuperscript{2+}
stores may stimulate PLC activity earlier than the PKC-medi-
ated suppression and contribute to the formation of the rising
phases of Ca\textsuperscript{2+} signaling. Complex DAG actions and cytosolic
Ca\textsuperscript{2+} concentration changes may underlie the spatio-tempo-
raly organized Ca\textsuperscript{2+} dynamics induced by IP\textsubscript{3}-mobilizing
agonists.

The molecular mechanisms by which DAG activates PLC
remain to be determined. First, what is the molecular target of
DAG? Blockade of OAG-induced Ca\textsuperscript{2+} oscillation by PP2 im-

Grimaldi et al. (28) recently reported the finding that OAG
application to glial cells induced slow Ca\textsuperscript{2+} oscillation that
was dependent on the extracellular Ca\textsuperscript{2+} concentration. They
showed that the OAG-induced Ca\textsuperscript{2+} oscillation was caused by
plies involvement of Src family protein tyrosine kinases (PTKs). However, because we were unable to detect any significant effect of DAG on Src (one of the Src family PTKs) activity in vitro (data not shown), Src is unlikely to be a direct target of DAG in the DAG-mediated PLC activation pathway. Thus, DAG may directly activate other DAG-binding proteins that lead to Src family PTK activation. Second, what type of PLC is activated by DAG? At least 12 mammalian PLC isoforms have been identified to date. They are PLCβ1–4, PLCγ1–2, PLCδ1–4, PLCε, and PLCζ (35, 36), and one of them, PLCε, has
FIG. 5. Effect of kinase activation and inhibition on OAG-induced Ca$^{2+}$ oscillation. A, acute application of PMA (1.0 μM), a PKC activator, did not evoke Ca$^{2+}$ oscillation in COS-7 cells. B, a serine-threonine kinase inhibitor, staurosporine (100 nM), did not inhibit the OAG-induced Ca$^{2+}$ oscillation. The arrow indicates OAG application. C, a DAG-lipase inhibitor (RHC83267, 25 μM) did not abolish OAG-induced Ca$^{2+}$ oscillation. D, involvement of Src family tyrosine kinases in OAG-induced Ca$^{2+}$ oscillation. A Src family tyrosine kinase inhibitor, PP2 (1.0 μM), blocked the OAG-induced Ca$^{2+}$ oscillation, whereas the inactive analog PP3 (1.0 μM) did not. Staurosporine, PP2, or PP3 was applied 5 min before the recording, and Ca$^{2+}$ imaging was performed in the presence of each of the agents. Each experiment was performed at least three times, and representative data are shown here.

FIG. 6. Accumulation of DAG by a DAG-lipase inhibitor enhances the Ca$^{2+}$ response to ATP stimulation. A, under the conditions in which PKC activity in COS-7 cells was down-regulated, the Ca$^{2+}$ response to various ATP concentrations (0.05, 0.1, 0.3, and 0.5 μM) was measured in the presence (right panel) and absence (left panel) of 50 μM RHC80267. RHC80267 was applied 5 min prior to the measurement. To avoid Ca$^{2+}$ store depletion, the measurements were made in the presence of external 2 mM Ca$^{2+}$. B, percentage of cells that responded to ATP. The black bars and white bars represent RHC80267-treated cells and control cells (PMA-treated alone). A total of 825 control cells from seven distinct experiments and a total of 1395 RHC80267-treated cells from 11 experiments were analyzed. Asterisks indicate statistically significant differences compared with the control by Student’s t test: p = 0.013 (0.05 μM ATP), 0.013 (0.1 μM ATP), 0.004 (0.3 μM ATP), and 0.034 (0.5 μM ATP).
recently been reported to be activated by Ras (37). In view of the fact that the activity of RasGRP, the regulator of Ras, is increased by DAG (31), PLCe may be one of the candidates for the molecule responsible for the DAG-induced IP3 production. However, since our data suggested possible involvement of Src family PTK activity in the signaling is unknown. Another candidate is PLCγ, because PLCγ can be activated by Src family PTK-mediated phosphorylation (38–40). Because Src family PTKs are also involved in the activation of receptor-PLC signaling through G-protein tyrosine phosphorylation (41), the phosphorylation of such signaling proteins by Src family PTKs may underlie the molecular mechanism of the DAG-induced PLC activation.

In conclusion, we propose a novel biological function of DAG as an inducer of IP3 production through PLC activation. The novel signal cross-talk between DAG and the IP3-mediated signal pathway amplifies agonist-induced Ca2+ signaling in response to weak extracellular stimuli. Because IP3R-mediated Ca2+ release plays an important role in various physiological functions including synaptic plasticity, gene expression, proliferation, and development, the increased IP3 production via DAG-mediated signaling may affect numerous biological phenomena.

Acknowledgments—We thank all of the members of our laboratories, especially Drs. T. Uchiyama, N. Matsumoto, H. Zhou, Y. Tateishi, A. Z. Suzuki, and S. Ozaki for their technical help and fruitful discussions.

REFERENCES

1. Berridge, M. J., and Irvine, R. F. (1989) Nature 341, 197–205
2. Berridge, M. J., Lipp, P., and Bootman, M. D. (2000) Science 287, 1604–1605
3. Berridge, M. J., Bootman, M. D., and Lipp, P. (1998) Nature 395, 645–648
4. Bootman, M. D., Lipp, P., and Berridge, M. J. (2001) J. Cell Sci. 114, 2213–2222
5. Hollinger, S., and Hepler, J. R. (2002) Pharmacol. Rev. 54, 527–559
6. Chen, C. A., and Manning, D. R. (2001) Oncogene 20, 1643–1652
7. Rhee, S. G. (2001) Annu. Rev. Biochem. 70, 281–312
8. Patel, S., Joseph, S. K., and Thomas, A. P. (1999) Cell Calcium 25, 247–264
9. Taylor, C. W., Genazzani, A. A., and Morris, S. A. (1999) Cell Calcium 26, 237–251
10. Arai, M. (2000) Jpn. Heart J. 41, 1–13
11. Misquitta, C. M., Mack, D. P., and Grover, A. K. (1999) Cell Calcium 25, 277–290
12. Joseph, S. K., and Williamson, J. R. (1989) Arch. Biochem. Biophys. 273, 1–15
13. Nishibe, S. G. (2001) J. Biol. Chem. 276, 340–345
14. Venkatachalam, K., Van Rossum, D. B., Patterson, R. L., Ma, H. T., and Gill, D. L. (2002) Nat. Cell Biol. 4, E263–E272
15. Meyer, T., and Stryer, L. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 5051–5055
16. Berridge, M., Lipp, P., and Bootman, M. (1999) Curr. Biol. 9, R157–159
17. Michikawa, T., Hirota, J., Kawano, S., Hiraksa, M., Yamada, M., Furuichi, T., and Mikoshiba, K. (1999) Neuron 23, 799–808
18. Maruyama, T., Kanai, T., Nakade, S., Kanno, T., and Mikoshiba, K. (1997) J. Biochem. (Tokyo) 122, 498–505
19. Miller, S., Romano, C., and Cotman, C. W. (1995) J. Neurosci. 15, 6103–6109
20. Uchiyama, T., Yoshikawa, F., Hisada, A., Furuichi, T., and Mikoshiba, K. (2002) J. Biol. Chem. 277, 8106–8113
21. Gryniewicz, G., Poenie, M., and Tsien, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
22. Preiss, J., Loomis, C. R., Bishop, W. R., Stein, R., Niedel, J. E., and Bell, R. M. (1986) J. Biol. Chem. 261, 8597–8600
23. Van Veldhoven, P. P., and Bell, R. M. (1988) Biochim. Biophys. Acta 959, 185–196
24. Hofmann, T., Obukhov, A. G., Schaefer, M., Harteneck, C., Gudermann, T., and Schultz, G. (1999) Nature 397, 259–263
25. Okada, T., Inoue, R., Yamazaki, K., Maeda, A., Kurokaki, T., Yamakuni, T., Tanaka, I., Shimizu, S., Ikemura, K., Imoto, K., and Mori, Y. (1999) J. Biol. Chem. 274, 27359–27370
26. Trebak, M., Vazquez, G., Bird, G. S., and Putney, J. W., Jr. (2003) Cell Calcium 33, 451–461
27. Zhang, L., and Saffer, D. (2001) J. Biol. Chem. 276, 13331–13339
28. Grimaldi, M., Maratos, M., and Verma, A. (2003) J. Biol. Chem. 278, 4737–4745
29. Andoh, T., Itah, H., Higashi, S., Saito, Y., Ishiwa, D., Kamiya, Y., and Yamada, Y. (2004) Brain Res. 1013, 125–133
30. Meijers, H. J., Palfrey, H. C., Hirning, L. D., and Miller, R. J. (1987) J. Neurosci. 7, 1198–1206
31. Ebinu, J. O., Bottorff, D. A., Chan, E. Y., Stang, S. L., Dunn, R. J., and Stone, J. C. (1998) Science 280, 1082–1086
32. Gordon, S. P., Downing-Park, J., Tam, R., and Zimmerman, A. L. (1995) Biophys. J. 69, 409–417
33. Cray, J. I., Dean, D. M., Nguiragwel, W., Kurshan, P. T., and Zimmerman, A. L. (2000) J. Gen. Physiol. 116, 753–768
34. Yue, C., Ku, C. Y., Liu, M., Simon, L. M., and Sanborn, B. M. (2000) J. Biol. Chem. 275, 30220–30225
35. Rebecchi, M. J., and Pentyala, S. N. (2000) Physiol. Rev. 80, 1291–1335
36. Fukami, K. (2002) J. Biochem. (Tokyo) 131, 293–299
37. Kelley, G. G., Reks, S. E., Ondrako, J. M., and Smrcka, A. V. (2001) EMBO J. 20, 743–754
38. Nishibe, S., Wahl, M. I., Hernandez-Sotomayer, S. M., Tonks, N. K., Rhee, S. G., and Carpenter, G. (1996) Science 250, 1253–1256
39. Liao, F., Shin, S. H., and Rhee, S. G. (1993) Biochem. Biophys. Res. Commun. 191, 1028–1033
40. Oezdener, F., Dangelmaier, C., Ashby, B., Kunapuli, S. P., and Daniel, J. L. (2002) Mol. Pharmacol. 62, 672–679
41. Unemori, H., Inoue, T., Kume, S., Seki, N., Nagao, M., Itah, H., Nakanishi, S., Mikoshiba, K., and Yamamoto, T. (1997) Science 276, 1878–1881
Amplification of Ca^{2+} Signaling by Diacylglycerol-mediated Inositol 1,4,5-Trisphosphate Production
Chihiro Hisatsune, Kyoko Nakamura, Yukiko Kuroda, Takeshi Nakamura and Katsuhiko Mikoshiba

J. Biol. Chem. 2005, 280:11723-11730.
doi: 10.1074/jbc.M409535200 originally published online January 6, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M409535200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2005/01/12/M409535200.DC1

This article cites 41 references, 18 of which can be accessed free at http://www.jbc.org/content/280/12/11723.full.html#ref-list-1