We have investigated the mechanisms involved in H$_2$O$_2$-mediated phospholipase D (PLD) activation in Swiss 3T3 fibroblasts. In the presence of vanadate, H$_2$O$_2$ induced tyrosine phosphorylation of PLD as well as the platelet-derived growth (PDGF) factor receptor, protein kinase Ca (PKC$_\alpha$), and a 62-kDa protein in rat brain PLD1 (rPLD1) immune complexes. PDGF also induced tyrosine phosphorylation of PLD, but this was abolished by catalase, indicating that it was mediated by H$_2$O$_2$ generation. Interestingly, PLD was found to be constitutively associated with the PDGF receptor and PKC$_\alpha$. Stimulation by H$_2$O$_2$ showed a concentration- and time-dependent tyrosine phosphorylation of the proteins in rPLD1 immunoprecipitates and activation of PLD in the cells. Pretreatment of the cells with the protein-tyrosine kinase inhibitors genistein and herbimycin A resulted in a concentration-dependent inhibition of H$_2$O$_2$-induced tyrosine phosphorylation and PLD activation. Activation of PLD by H$_2$O$_2$ was also inhibited dose-dependently by the PKC inhibitors Ro-31-8220 and calphostin C. Down-regulation of PKC by prolonged treatment with 4β-phorbol 12-myristate 13-acetate also abolished H$_2$O$_2$-stimulated PLD activity. H$_2$O$_2$ or vanadate alone did not induce tyrosine phosphorylation of proteins in the rPLD1 immune complex or PLD activation. Reduction of intracellular H$_2$O$_2$ levels by pretreatment of the cells with catalase dramatically abrogated tyrosine phosphorylation of proteins in the rPLD1 immune complex and PLD activation, suggesting the potential role of intracellular H$_2$O$_2$ in H$_2$O$_2$-mediated PLD signaling. Taken together, these results suggest that both protein-tyrosine kinase(s) and protein kinase C participate in H$_2$O$_2$-induced PLD activation in Swiss 3T3 cells.

Cell-surface receptor-mediated hydrolysis of phosphatidylcholine and other membrane phospholipids by phospholipase D (PLD)$^1$ has been recognized as a mechanism of signal transduction in mammalian cells (1, 2). PLD hydrolyzes phosphatidylcholine to choline and phosphatidic acid. The latter acts as a second messenger and can be further converted to other messenger molecules, namely 1,2-diacylglycerol and lysophosphatidic acid. PLD activation following interaction of agonists with G protein-coupled receptors and receptors with tyrosine kinase activity has been observed in many cells and tissues (3, 4). Although the precise physiological function of PLD in cells is poorly understood, receptor-stimulated PLD activity has been implicated in a broad range of physiological responses (1–5). These include rapid responses (secretion and superoxide generation) as well as long-term responses (proliferation, differentiation, and immune responses). PLD is known to be activated via multiple pathways involving G proteins, protein kinase C (PKC), protein-tyrosine kinases, and Ca$^{2+}$(1).

Recently, H$_2$O$_2$ was reported to stimulate PLD activity in endothelial cells (6), NIH 3T3 fibroblasts (7), and PC12 cells (8). Reactive oxygen species such as hydrogen peroxide and superoxide have been shown to be generated in a variety of cells stimulated with cytokines, growth factors, and agonists of G protein-linked receptors, and it has been suggested that they may act as second messengers (9). It was also reported that oxidant stress due to H$_2$O$_2$ induced tyrosine phosphorylation of cell proteins that was strongly potentiated by combination with vanadate (10). The mechanism was believed to be due, at least in part, to inhibition of protein-tyrosine phosphatase (10) or activation of protein-tyrosine kinase activity (11) or both. H$_2$O$_2$ has been reported to enhance tyrosine phosphorylation of the platelet-derived growth factor (PDGF) receptor, the epidermal growth factor receptor, Src kinase, and mitogen-activated protein kinase, leading to activation of gene expression including c-fos, c-myc, c-jun, NF-κB, and AP-1 (12–16). Tyrosine phosphorylation has also been implicated in PLD activation mediated by epidermal growth factor (17), PDGF (18), pervanadate (19, 20), fMet-Leu-Phe (21), and immunoglobulin E (22). Furthermore, inhibitors of protein-tyrosine phosphatases such as peroxides of vanadate stimulate PLD activity by G protein-independent (19) and -dependent (23) mechanisms. Recently, it was reported that, in response to stimulation by peroxides of vanadate, human brain PLD1 was tyrosine-phosphorylated and associated with several undefined, tyrosine-phosphorylated proteins in HL-60 cells (20).

We have cloned and characterized a rat brain phospholipase D isozyme (rPLD1) (24, 25). In the present study, we have investigated the effect of hydrogen peroxide as a pharmacologic agent on tyrosine phosphorylation and activation of rPLD1 in Swiss 3T3 fibroblasts. We report that H$_2$O$_2$ induces tyrosine phosphorylation of PLD as well as the PDGF receptor, PKC$_\alpha$, and an unknown protein of 62 kDa in a rPLD1 immune complex. Further results suggest that protein-tyrosine kinase and PKC may participate in H$_2$O$_2$-mediated PLD activation in Swiss 3T3 cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Swiss 3T3 cells were purchased from American Type Culture Collection. Fetal bovine serum and Dulbecco's modified Eagle's...
medium were from Life Technologies, Inc. Protein A-Sepharose and the ECL Western blotting detection kit were from Amersham Pharmacia Biotech. Nonidet P-40, sodium orthovanadate (Na$_3$VO$_4$), H$_2$O$_2$, and all other reagents were from Sigma. Affi-Gel 15 was from Bio-Rad. The monoclonal anti-phosphotyrosine 4G10 antibody was obtained from Upstate Biotechnology, Inc. Polyclonal antibody to the PDGF receptor was from Oncogene Science Inc. and Upstate Biotechnology, Inc. Poly-
den was from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD).

PDGF-BB was from Boehringer Mannheim.

Swiss 3T3 Cell Culture—Swiss 3T3 fibroblasts were grown in Dul-
becco's modified Eagle's medium supplemented with 10% heat-inacti-
vated fetal bovine serum, 100 units/ml penicillin, and 100 
mg/ml streptomy-
cin in a humidified atmosphere containing 5% CO$_2$ at 37 °C. Cells

were from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD).

radish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG were
from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD).

with 20 mM HEPES (pH 7.2) containing 1% Triton X-100, 10% glycerol,
and 1% Nonidet P-40.

were probed with anti-rPLD1, anti-Tyr(P), anti-PDGF re-
ceptor, and PKC$_\alpha$ antibodies as the first antibody. Blots were washed in 20 mM
Heps (pH 7.2) containing 1% Triton X-100, 10% glycerol, 50 mM NaF, 1 mM
Na$_3$VO$_4$, 10 
mg/ml leupeptin, 10 
mg/ml aprotinin, and 1
mM phenylmethylsulfonyl fluoride. After incubation for 30 min in an
ice bath, cell lysates were cleared by centrifugation at 15,000 rpm in an
Eppendorf microcentrifuge for 10 min at 4 °C. Protein concentrations
were determined using the Bio-Rad protein assay. For immunoprecipi-
tation, equal protein aliquots (500 
g of total protein) were used, and
the supernatants were precleared with preimmune IgG and protein
A-Sepharose for 30 min at 4 °C. Precleared cell lysates were incubated with the indicated antibodies (anti-rPLD1, anti-Tyr(P), anti-PDG-
F receptor, and anti-PKC$_\alpha$) and with 60 
ul of a 1:1 slurry of protein
A-Sepharose for 4 h at 4 °C. The immunoprecipitates were collected by
centrifugation, washed five times with buffer containing 20 mM tris
(pH 7.5), 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 2 mM Na$_3$VO$_4$, 10
% glycerol, and 1 % Nonidet P-40.

Immunoblot Analysis—Immune complexes were boiled in SDS sam-
ple buffer and subjected to 4–12% gradient SDS-polyacrylamide gel
electrophoresis. Proteins were transferred to a polyvinylidene diflu-
oride membrane (Immobilon-P, Millipore Corp.), and blots were ince-
bated for 20 min with 20 mM Tris (pH 7.6), 150 mM NaCl, and 0.05%
(v/v) Tween 20 containing 5% (v/v) nonfat dried milk (for detection of
PLD, the PDGF receptor, and PKC$_\alpha$) or with phosphate-buffered saline
containing 3% nonfat dried milk (for detection of Tyr(P)). Immunoblot-
s were probed with anti-Tyr(P), anti-rPLD1, anti-PDG-F receptor,
and anti-PKC$_\alpha$ antibodies as the first antibody. Blots were washed in 20 mM
Tris (pH 7.6), 150 mM NaCl, and 0.05% (v/v) Tween 20. Depending on
the origin of the primary antibodies, anti-mouse or anti-rabbit IgG was
used for detection using the ECL reaction.

Phospholipase D Assay—Cells were plated in 60-mm tissue culture
plates. The cells were serum-starved in Dulbecco's modified Eagle's
medium supplemented with 0.5% fatty acid-free bovine serum albumin
for 24 h before the start of the assay. The procedures for the assay were
those of Hess et al. (26). For the final 20 h of serum starvation, the cells
were labeled with 1 
cl/ml [9,10-3H]myristic acid. The cells were
were washed as described (26) and reincubated with 0.3% butan-1-ol for
10 min. The cells were then treated with H$_2$O$_2$ or PDGF for the indicated
times, and phosphatidylbutanol formation was measured as described
(26). Radioactivity incorporated into total phospholipids was measured
and used to normalize the results.

RESULTS

Hydrogen Peroxide and PDGF Induce Tyrosine Phosphoryla-
tion of PLD and Proteins in rPDL1 Immune Complexes in
Swiss 3T3 Fibroblasts—We initially investigated the role of
tyrosine phosphorylation in the activation of PLD in Swiss 3T3
fibroblasts. Serum-starved cultures of the cells were exposed to
H$_2$O$_2$ (200 
M) or PDGF-BB (100 ng/ml) after pretreatment
with orthovanadate (100 
M) for 20 min. Cell lysates were
prepared and subjected to immunoprecipitation with antibody
against either rPLD1 or Tyr(P). The presence of tyrosine-phos-
phorylated proteins and rPLD1 in the immunoprecipitates was
monitored by successive immunoblotting. As shown in Fig. 1A,
anti-rPLD1 antibody precipitated tyrosine-phosphorylated pro-
teins of ~120 kDa (major band) and 185, 85, and 62 kDa in the
H$_2$O$_2$-stimulated cells (lane 2). In PDGF-stimulated cells, pro-
teins of ~185 kDa (major band) and 120 kDa were found (lane
3). In contrast, no tyrosine-phosphorylated proteins (except for
IgG chains) could be detected in unstimulated cells (lane 1).

To investigate whether the 120-kDa tyrosine-phosphorylated
protein was PLD itself or an associated protein, cell lysates
were immunoprecipitated with anti-rPLD1 antibody and sub-
jected to Western blot analysis using anti-rPLD1 antibody (Fig.
1B). Equal amounts of PLD were immunoprecipitated from
control and agonist-treated cells. The electrophoretic mobility
of PLD correlated with that of the 120-kDa phosphotyrosine
protein (Fig. 1B). These experiments were also performed using
the reverse protocol. The tyrosine-phosphorylated proteins
were immunoprecipitated with anti-Tyr(P) antibodies, and the
amount of PLD in the Tyr(P) immune complex was assessed by
Western blotting (Fig. 1C). The results show the presence of
PLD in the anti-Tyr(P) immunoprecipitates from the H$_2$O$_2$-
stimulated cells (lane 2), whereas PLD was barely detectable in
the immunoprecipitates from PDGF-stimulated cells (lane 3).

The amount of PLD correlated with its extent of tyrosine
phosphorylation as shown in Fig. 1A. Taken together, these results
indicate that PLD is tyrosine-phosphorylated on stimulation of
Swiss 3T3 cells with H$_2$O$_2$ and is associated with several ty-
rosine-phosphorylated proteins including p185, p85, and p62.
To test whether p85 was PKC$_\alpha$, lysates from control or H$_2$O$_2$-
treated cells were immunoprecipitated with anti-PKC$_\alpha$ anti-
bodies, and the immune complexes were blotted for tyrosine-
phosphorylated proteins. As shown in Fig. 1D, a phosphoprotein corresponding to the PKC isoform was
found in the immunoprecipitates from the treated cells. When
the reciprocal experiment was performed (Fig. 1E), PKC$_\alpha$ was
found in the Tyr(P) immune complex. Thus, p85 is almost certainly PKC$_\alpha$.

PLD Is Constitutively Associated with the PDGF Receptor
and PKC$_\alpha$—Next, we attempted to identify the 185-, 85-, and
62-kDa tyrosine-phosphorylated proteins in the anti-rPLD1
immunoprecipitates. The proteins were examined using anti-
bodies to several known signaling molecules of similar molecu-
lar masses including the PDGF receptor, PKC, and Src. These
were also chosen since it has been reported recently that H$_2$O$_2$
induces tyrosine phosphorylation of the PDGF receptor, Src,
and PKC$_\alpha$ (17, 27). Co-immunoprecipitation experiments were
used to investigate the possibility that H$_2$O$_2$ (or PDGF) induces
tyrosine phosphorylation of these proteins and their associa-
tion with PLD. The results are shown in Figs. 1 (D–G) and 2. The
results show the association between PLD and PKC$_\alpha$. As shown in Fig. 1 (D–G), PKC$_\alpha$ was present in the anti-rPLD1 immunoprecipitates, and rPLD1 was present in the anti-PKC$_\alpha$ immune complexes. Surpris-
ingly, PLD was constitutively associated with the PDGF
receptor and PKC$_\alpha$ since the association was not altered by H$_2$O$_2$ or
PDGF (lanes 2 and 3 in Fig. 1 (F and G) and Fig. 2) or omission of vanadate (data not shown).

Phosphotyrosine analysis revealed a H2O2-induced tyrosine phosphorylation of the ~62-kDa protein (Fig. 1A), and work by others has demonstrated a physical association between the activated PDGF receptor and pp60c-src (34, 35). We therefore investigated whether or not the 62-kDa protein phosphorylated by H2O2 treatment was c-Src. We confirmed the presence of Src in Swiss 3T3 fibroblasts using immunoprecipitations and Western blot analysis. However, immunoprecipitations using anti-Src or anti-rPLD1 antibody and subsequent immunoblotting with anti-rPLD1 and anti-Src antibodies, respectively, did not reveal any 62-kDa protein (data not shown). Thus, the tyrosine-phosphorylated 62-kDa protein associated with PLD is not c-Src.

**Dose Dependence of Tyrosine Phosphorylation and Activation of PLD by H2O2**—To examine the dose-dependent tyrosine phosphorylation of protein species by H2O2, quiescent Swiss 3T3 fibroblasts were pretreated with vanadate and then treated without or with various concentrations of H2O2 for 10 min. The cell lysates were immunoprecipitated with anti-rPLD1 antibody and subjected to immunoblotting with anti-Tyr(P) antibody. No tyrosine-phosphorylated protein bands were detected in control cells (apart from IgG chains). A small increase of tyrosine phosphorylation was induced with 100 μM H2O2, whereas 200 μM, 500 μM, and 1 mM H2O2 dramatically induced tyrosine phosphorylation of several proteins (Fig. 3A). Strong tyrosine phosphorylation of a 120-kDa protein was detected as well as tyrosine phosphorylation of 185-, 85-, and 62-kDa proteins. Tyrosine phosphorylation of these proteins in the rPLD1 immune complex was dose-dependent, reaching a maximal level at 500 μM to 1 mM H2O2. Since Figs. 1 and 2 indicated that the 120-, 185-, and 85-kDa proteins were PLD, the PDGF receptor, and PKCa, respectively, the blots were stripped and reprobed with the anti-rPLD1, anti-PDGF receptor, and anti-PKCa antibodies (Fig. 3A). This confirmed the presence of PLD, the PDGF receptor, and PKCa in all lanes. However, the amounts were not altered by H2O2 stimulation.
In Swiss 3T3 cells prelabeled with \(^{3}H\)myristate and preincubated with vanadate and 0.3% butanol, \(H_2O_2\) stimulated \([3H]\)phosphatidylbutanol (PtdBut) formation in a dose-dependent manner (Fig. 3B). These results indicate a correlation between effects of \(H_2O_2\) on tyrosine phosphorylation and activation of PLD.

**Time Dependence of Tyrosine Phosphorylation and Activation of PLD by \(H_2O_2\)—** To investigate the time course of the \(H2O_2\)-induced tyrosine phosphorylation, quiescent Swiss 3T3 cells were pretreated with vanadate and then treated with 500 \(\mu M\) \(H_2O_2\) for the indicated times, and the cell lysates were immunoprecipitated with anti-rPLD1 antibody and subjected to Western blotting with anti-Tyr(P) antibody. Tyrosine phosphorylation of the 120-kDa PLD protein band was weakly detected after 5 min of \(H_2O_2\) stimulation (Fig. 4A) and clearly detectable after 20 min. At and after 20 min, other tyrosine-phosphorylated proteins were detected. Interestingly, the time courses of tyrosine phosphorylation of the proteins were different. Tyrosine phosphorylation of PLD was greatest at 20 min of \(H_2O_2\) stimulation, but tyrosine phosphorylation of the PDGF receptor, PKCa and the unidentified 62-kDa protein was greatest at 50 min. To define the time course of \(H_2O_2\)-induced PLD activation, \([3H]\)PtdBut formation was measured in \([3H]\)myristate-labeled cells (Fig. 4B). PLD activation by \(H_2O_2\) was rapid for 10 min and continued at a slower rate up to 50 min, whereas \(H_2O_2\)-induced tyrosine phosphorylation of PLD peaked at 20 min. These results suggest that tyrosine phosphorylation is only partly involved in PLD activation.

**Combined Effect of \(H_2O_2\) Plus Vanadate on Tyrosine Phosphorylation and Activation of PLD—** Since the combined use of hydrogen peroxide and vanadate was reported to trigger strong tyrosine phosphorylation in various cell types (19, 28–30), this was used in our experiments. Because of literature discrepancies in the effects of \(H_2O_2\) alone on tyrosine phosphorylation (10, 29, 31, 32), we investigated the separate effects of \(H_2O_2\) and vanadate on tyrosine phosphorylation in Swiss 3T3 cells (Fig. 5). Cells were treated with or without 100 \(\mu M\) Na\(_2\)VO\(_4\) or 500 \(\mu M\) \(H_2O_2\) for 20 min. PLD activity was assayed under the same conditions. For the tyrosine phosphorylation experiments, cell lysates were immunoprecipitated with anti-rPLD1 antibody and immunoblotted with anti-phosphotyrosine antibody. When the cells were treated with \(H_2O_2\) or Na\(_2\)VO\(_4\) alone, no tyrosine phosphorylation was detected. However, treatment with \(H_2O_2\) plus vanadate induced marked tyrosine phosphorylation of proteins in the immune complex (Fig. 5A). In addition, PLD activation also was markedly increased by \(H_2O_2\) plus vanadate, but was very minimally affected by \(H_2O_2\) or vanadate alone (Fig. 5B).
to phosphorylate and activate PLD, cells were pretreated with various concentrations of genistein and herbimycin A, inhibitors of protein-tyrosine kinase, prior to stimulation with 500 μM H₂O₂ for 20 min. Cell lysates were then subjected to immunoprecipitation using anti-rPLD1 antibody and Western blotting (Blot) with anti-Tyr(P) antibody. Fig. 6 (A and B) shows that pretreatment with genistein or herbimycin A inhibited the H₂O₂-induced tyrosine phosphorylation of proteins in the rPLD1 immune complex in a concentration-dependent fashion. We also examined the effects of the inhibitors on PLD activation by H₂O₂. Both also attenuated the H₂O₂-mediated increase in PLD activation in a dose-dependent manner (Fig. 6, C and D). However, like the tyrosine phosphorylation, the inhibition was only partial. These results support the view that H₂O₂-mediated protein tyrosine phosphorylation is involved in PLD activation in Swiss 3T3 fibroblasts.

Involvement of PKC in the Regulation of PLD by H₂O₂—To investigate the possible role of PKC in H₂O₂-stimulated PLD activity, we applied two approaches, namely the use of PKC inhibitors and depletion of the enzyme by prolonged exposure of cells to 4β-phorbol 12-myristate 13-acetate. We tested Ro 31-8220 and calphostin C, which have been reported to be potent and selective PKC inhibitors (33). Ro 31-8220 competes with ATP for binding to PKC, whereas calphostin C interacts with the regulatory domain to compete with diacylglycerol and phorbol esters. Cells were preincubated with increasing concentrations of the inhibitors for 40 min prior to H₂O₂ stimulation. To exclude a possible role of PKC in protein tyrosine phosphorylation (34), we examined tyrosine phosphorylation of proteins in H₂O₂-stimulated cells in the absence or presence of the inhibitor. Immunoprecipitation with anti-rPLD1 antibody and Western blotting with anti-phosphotyrosine antibody demonstrated that Ro 31-8220 had a negligible effect on protein tyrosine phosphorylation in the rPLD1 immune complex in cells stimulated with H₂O₂ (500 μM) for 20 min (data not shown). The
possibility that the effect of H2O2 on PLD stimulation involves PKC activation was then tested utilizing Ro 31-8220 and calphostin C. The stimulation of PLD activity by H2O2 (500 μM, 20 min) was inhibited by Ro 31-8220 and calphostin C in a dose-dependent manner (Fig. 7, A and B), although the inhibition was not complete.

PKCa, -δ, -ε, and -ζ are present in Swiss 3T3 cells, and treatment of cells with 500 nM 4β-phorbol 12-myristate 13-acetate for 48 h differentially down-regulates the α, δ, and ε isozymes, but not PKCaζ (35). We therefore investigated the effect of PKC down-regulation on H2O2-stimulated PLD activity. As shown in Fig. 7C, the treatment itself enhanced PtdBut formation. However, the ability of H2O2 to promote PtdBut accumulation was almost completely abolished in the down-regulated cells. These results strongly support the conclusion that PKC is importantly involved in PLD activation by H2O2. Combination of genistein and Ro 31-8220 blocked PLD activation significantly, but not completely (Fig. 8), suggesting that another pathway may exist for regulation of PLD in Swiss 3T3 cells.

 Pretreatment with Catalase Abolishes Tyrosine Phosphorylation and PLD Activation Induced by PDGF—To assess the role of H2O2 as the reactive oxygen species responsible for PDGF-induced tyrosine phosphorylation, we examined the effect of catalase. This highly reactive enzyme has been widely used to block the biological activities of H2O2 since it is taken up by cells (13). As shown in Fig. 9A, pretreatment of Swiss 3T3 cells with catalase (0.1 or 1 μg/ml) for 1 h abrogated tyrosine phosphorylation of proteins in rPLD1 immunoprecipitates induced by PDGF (200 ng/ml, 20 min) and also inhibited tyrosine phosphorylation of lysate proteins in general (Fig. 9B). As expected, catalase also inhibited the effects of exogenous H2O2 on PLD activity (data not shown). These results demonstrate the role of intracellular H2O2 in the PDGF effect on PLD phosphorylation and provide no evidence for direct phosphorylation of the enzyme by the PDGF receptor.

PDGF treatment, with or without vanadate preincubation, stimulated PLD activity, and this was partially inhibited by catalase (Fig. 10). These data suggest a role for H2O2, but also...
for another pathway, probably one involving phospholipase C and PKC (1, 2).

DISCUSSION

It has been reported that exogenously added H$_2$O$_2$ stimulates PLD activity in bovine pulmonary artery endothelial cells, NIH 3T3 fibroblasts, and PC12 cells (6–8), but the underlying mechanism(s) remains poorly understood. In the present study, we have examined the effects of H$_2$O$_2$ and PDGF on protein tyrosine phosphorylation and activation of PLD to understand the mechanism(s) involved in the regulation of the enzyme in Swiss 3T3 fibroblasts. Several recent studies have indicated that a protein-tyrosine kinase(s) participates in PLD activation via an unidentified mechanism (37). Here we report that the combined use of vanadate and H$_2$O$_2$ induced strong tyrosine phosphorylation of PLD and that PLD was constitutively associated with the PDGF receptor and PKC$_\alpha$, which were also tyrosine-phosphorylated by H$_2$O$_2$. These findings support a recent report showing that exposure of HL-60 cells to peroxides increases tyrosine phosphorylation of the hPLD1 isoform of PLD and of several other proteins in the hPLD1 immune complex (20). Interestingly, vanadate or hydrogen peroxide added alone to intact Swiss 3T3 cells had no effects on tyrosine phosphorylation of proteins in the rPLD1 immune complex. This is in contrast with the known inhibitory effects of vanadate on protein-tyrosine phosphatase activity in vitro (38). This discrepancy may be attributed to the lower concentration of vanadate used in the present study. It appears that in certain cell types, the rate of intracellular accumulation of vanadate is relatively slow, requiring either prolonged incubations or the use of relatively high vanadate concentrations (39, 40).

As expected, the protein tyrosine phosphorylation induced by H$_2$O$_2$ plus vanadate was blocked by pretreatment with the protein-tyrosine kinase inhibitors genistein and herbimycin A. The associated PLD activation was also inhibited by these agents. The inhibitions of protein tyrosine phosphorylation and PLD activation (Fig. 6) were only partial, but showed some correlation, suggesting a possible role of protein tyrosine phosphorylation in H$_2$O$_2$-induced PLD stimulation. Hydrogen peroxide-induced tyrosine phosphorylation reached a maximal level 20 min after H$_2$O$_2$ treatment, whereas PLD activation

Fig. 7. Effect of PKC inhibitors and PKC down-regulation on H$_2$O$_2$-induced PLD activation. [H]Myristate-labeled Swiss 3T3 cells were pretreated with the indicated concentrations of Ro 31-8220 (A) or calphostin C (B) for 40 min and vanadate (100 $\mu$M) for 20 min prior to stimulation with H$_2$O$_2$ (500 $\mu$M) for 20 min. For down-regulation of PKC (C), the cells were pretreated without or with 500 $\mu$M 4$\beta$-phorbol 12-myristate 13-acetate for 48 h and then pretreated with vanadate prior to stimulation without (C) or with H$_2$O$_2$ for 20 min. The radioactivity incorporated into PtdBut was measured as described under “Experimental Procedures.” Data are representative of two experiments conducted in duplicate.

Fig. 8. Effect of combination of genistein and Ro 31-8220 on PLD activation by H$_2$O$_2$. [H]Myristate-labeled cells were pretreated with genistein (100 $\mu$M) and/or Ro 31-8220 (10 $\mu$M) for 40 min and with vanadate (100 $\mu$M) for 20 min prior to stimulation with H$_2$O$_2$ (100 $\mu$M) for 20 min. The radioactivity incorporated into PtdBut was measured as described under “Experimental Procedures.” Data are representative of two experiments conducted in duplicate.
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continued progressively thereafter (Fig. 4, A and B). This suggests that, although the initial tyrosine phosphorylation may be partly involved in H$_2$O$_2$-induced PLD activation, other mechanisms are responsible for the later increase. A problem with the interpretation of these experiments is that the effects of the inhibitors on tyrosine phosphorylation were only partial. Thus, the full contribution of this phosphorylation to PLD activation cannot be determined.

Although a recent study reported that H$_2$O$_2$-induced PLD activation was not linked to PKC activation in PC12 cells (8), our studies using selective PKC inhibitors (Ro 31-8220 and calphostin C) and down-regulation of PKC suggest that H$_2$O$_2$-induced PLD activation is partly dependent on PKC in Swiss 3T3 cells. This may reflect a difference in the pathways for H$_2$O$_2$-mediated PLD stimulation in these cells. As noted above, tyrosine phosphorylation of PLD by H$_2$O$_2$ is not well correlated temporally with activation of the enzyme. Thus, another activation mechanism involving tyrosine phosphorylation of another protein seems likely. The possibility that PKCa is involved is strong because this kinase is tyrosine-phosphorylated in response to H$_2$O$_2$ (Fig. 1, D and E) and because of the effects of PKC inhibition and down-regulation (Fig. 7). Furthermore, the tyrosine phosphorylation of PKC isoforms induced by H$_2$O$_2$ in COS-7 cells has been shown to be associated with an increase in activity (41). However, it should be recognized that H$_2$O$_2$ might also regulate PLD by a mechanism involving PKC, but not tyrosine phosphorylation. Such a possibility is suggested by the large effect of PKC down-regulation (Fig. 7 C) and the results of combining a tyrosine kinase inhibitor with a PKC inhibitor (Fig. 8).

It has been shown that H$_2$O$_2$ directly inhibits protein-tyrosine phosphatase activity (42) and that all protein-tyrosine phosphatases contain an essential sulfhydryl group that is crucial for activity and susceptible to oxidation (43). Thus, it seems probable that a protein-tyrosine phosphatase is a target of H$_2$O$_2$ in the present study. Inactivation of protein-tyrosine phosphatases would be expected to result in increased tyrosine phosphorylation since their specific activities in vitro are 10–1000 times those of protein-tyrosine kinases (44). In the presence of H$_2$O$_2$, orthovanadate is oxidized to pervanadate (45), which could facilitate its entry into cells. Vanadate seems to play a dual role in potentiating H$_2$O$_2$ effects on protein tyrosine phosphorylation. After entry into cells, most of the vanadate is reduced to the vanadyl(IV) ion that forms tight complexes with proteins (46). As such, vanadyl could inhibit protein-tyrosine phosphatases in a way similar to its capacity to inhibit alkaline phosphatase activity (47). In addition, vanadyl, like orthovanadate, could directly activate protein-tyrosine kinases (48, 49).

In Swiss 3T3 cells, PDGF also induced tyrosine phosphorylation of PLD, and a role for H$_2$O$_2$ was shown by the inhibitory effects of catalase on tyrosine phosphorylation (Fig. 9 A) and activity (Fig. 10) of PLD. Recent work in A431 and vascular smooth muscle cells has shown the requirement for generation of H$_2$O$_2$ for epidermal growth factor and PDGF signal transduction (9, 50). The growth factor-stimulated rise in H$_2$O$_2$ may serve to transiently inactivate intracellular protein-tyrosine phosphatases, allowing for a temporary alteration in the kinase-phosphatase balance and enhancing the action of the growth factors. The partial effect of catalase on PLD activation by PDGF is not unexpected because the ability of the growth factor to activate PLD via phosphoinositide-specific phospholipase C and PKC in Swiss 3T3 is well established (1, 2), and

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**FIG. 9.** Effect of catalase on PDGF-induced tyrosine phosphorylation. Quiescent cells were pretreated with the indicated concentrations of catalase for 1 h and pretreated with vanadate (100 μM) for 20 min prior to stimulation with PDGF (200 ng/ml) for 20 min. Cell lysates were subjected to immunoprecipitation (IP) using anti-rPLD1 antibody and Western blot analysis (Blot) with anti-Tyr(P) antibody (A) or to direct immunoblotting using anti-Tyr(P) antibody (B). The blots shown are representative of two experiments.

**FIG. 10.** Effect of vanadate or catalase on PLD activation by PDGF. [3H]Myristate-labeled cells were pretreated with or without catalase (1 ng/ml) for 1 h and pretreated with or without vanadate (100 μM) for 20 min prior to stimulation with PDGF (100 ng/ml) for 20 min. The radioactivity incorporated into PtdBut was measured as described under “Experimental Procedures.” Data are representative of two experiments conducted in duplicate.
catalase appeared to produce little inhibition of autophosphorylation of the PDGF receptor (Fig. 9B). The large effect of catalase on the tyrosine phosphorylation of cell proteins induced by PDGF is in agreement with findings with epidermal growth factor in A431 cells (9) and indicates an important role for H₂O₂ in this phosphorylation.

An interesting finding of the present study was the observation that rPLD1 immunoprecipitates contained the PDGF receptor and PKCα. Likewise, the PDGF receptor and PKCα immunoprecipitates contained rPLD1. Surprisingly, PDGF or H₂O₂ treatment of the cells did not alter the association of rPLD1 with the PDGF receptor or the association of rPLD1 with PKCα (Figs. 1 (F and G) and 2), and the associations occurred in the absence of vanadate. These data suggest that rPLD1 is constitutively associated with the receptor and the protein kinase and that this association is not influenced by tyrosine phosphorylation. We have also observed that rPLD1 and PKCα are associated in S9 cells and that the interaction is enhanced by treatment of the cells with phorbol ester.² Furthermore, direct interaction between these proteins is indicated by in vitro findings (25, 36). The association of rPLD1 with the PDGF receptor is somewhat surprising, although the fact that it is not enhanced by PDGF is to be expected since this PLD isoform has no recognizable SH2 or PTB domains (24, 36). Whether the association has functional significance remains to be determined.

It is clear from the present findings that, although H₂O₂ causes tyrosine phosphorylation of PLD, this cannot fully explain the activation of the enzyme, as shown by the difference in the time courses and the effects of PKC inhibitors. The possibility that tyrosine phosphorylation of PKCα or another regulatory protein is responsible for the activation of PLD needs further exploration, as does the significance of the apparent association of PLD with the PDGF receptor.

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