Phospholipase D (PLD) has been implicated in vesicle trafficking in the Golgi and hence secretion. In this study, we show that the secretion of matrix metalloproteinase-9 (MMP-9) from HT 1080 human fibrosarcoma cells was stimulated by phorbol 12-myristate 13-acetate in a time- and dose-dependent manner that involved protein kinase C. The phorbol ester also increased PLD activity in the cells. Evidence that PLD was involved in the stimulation of MMP-9 secretion was provided by the observations that the secretion of MMP-9 was stimulated by the introduction of short-chain phosphatidic acid (PA) into the growth medium and that inhibition of PA production by 1-propanol inhibited secretion. Using a short-chain diacylglycerol we excluded the possibility that MMP-9 secretion was induced by diacylglycerol formed by PA by phosphatidic acid phosphatase. Furthermore, propranolol, an inhibitor of this enzyme, had no effect on secretion induced by either phorbol 12-myristate 13-acetate or PA. The data presented here indicate that activation of protein kinase C increases MMP-9 secretion in HT 1080 cells and implicate PLD and PA formation in the effect.

ADP-ribosylation factors (ARFs) in their myristoylated and GTP-bound form are essential for COPI (coatomer) and clathrin coat assembly on the Golgi and for maintenance of this organelle (1–5). ARFs also activate phospholipase D (PLD), and it has been suggested that the Golgi-associated PLD mediates some of the effects of ARF on the Golgi (6). Recently, it has been shown that phosphatidic acid (PA), the product of phosphatidylcholine hydrolysis by PLD, promotes vesicular transport from the endoplasmic reticulum to the Golgi (7) and also budding of secretory vesicles from the trans-Golgi network (8, 9). Furthermore, protein kinase C has been suggested to participate in the control of vesicular trafficking to, through, and from Golgi components.

Secretion of matrix metalloproteinases (MMPs) from cancer cells is an important stage in the metastatic spread. MMPs hydrolyze collagen, a major component of the extracellular matrix, and allow the invasion of cancer cells from their primary site to the circulation and secondary sites (12–14). Various stimuli, including phorbol 12-myristate 13-acetate (PMA), have been shown to induce the secretion of MMP-9 from cancer cell lines (15) including the human fibrosarcoma line HT 1080 (16, 17). Focusing on the mechanisms regulating Golgi functions, we investigated the regulation of MMP-9 secretion from HT 1080 cells. PMA stimulated both MMP-9 secretion and PLD activity in a time- and dose-dependent manner. A role for PLD was indicated by the observation that inhibition of PA production blocked MMP-9 secretion. Furthermore, the addition of diocanoylphosphatidic acid (DOPA) induced high secretion of MMP-9. The effect of PMA seemed to be direct because the product of its hydrolysis by phosphatidic acid phosphatase (PAP) dioctanoylglycerol (DOG) had only a minor effect on secretion. Moreover, inhibition of PAP with propranolol had no effect on secretion induced by PMA. These findings and those to be reported elsewhere implicate ARF and PLD in the stimulation of MMP-9 secretion by protein kinase C (PKC).

**EXPERIMENTAL PROCEDURES**

**Materials**—Essentially fatty acid-free bovine serum albumin (BSA) and PMA were products of Sigma. Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, and all other supplements for growth media were purchased from Life Technologies, Inc. Lipids were purchased from Avanti Polar Lipids Inc. [9,10-3H]Myristic acid was a product of NEN Life Science Products. Polyacrylamide 10% zymogram gels, zymogram renaturing, and developing buffers were products of Novex. All organic solvents were of fine grade and were obtained from Fisher.

**Cell Culture**—Cells were maintained in DMEM, 10% fetal calf serum, 10 units/ml penicillin, and 10 μg/ml streptomycin (growth medium) at 37 °C and in 10% CO2 atmosphere. For PLD assays 8 × 10^5 cells were seeded in 60-mm plates and then allowed to grow for 24 h. Prior to the experiments, cells were serum-deprived for 18 h in DMEM, 0.1% BSA. For PLD assays, 35-mm plates were seeded with 3.5 × 10^5 cells. After 24 h, the cells were serum-deprived and labeled in DMEM, 0.1% BSA with 1 μCi/ml [3H]myristic acid for 18 h.

**PLD Assay**—The serum-deprived and labeled cells were washed with DMEM, 0.1% BSA, and following a 20-min preincubation in DMEM, 0.1% BSA, and 0.3% 1-butanol, they were stimulated with PMA at the concentrations and times given in the figure legends. Cells were washed with phosphate-buffered saline (1.68 mM KCl, 1.47 mM KH2PO4, 8.05 mM Na2HPO4, 137 mM NaCl) and 0.1% BSA, scraped with 1 ml of ice-cold CH3OH, and transferred into glass tubes. CHCl3 and 0.1 M HCl were added to a final ratio of 1:1:1. The lipid-containing lower phase was collected, dried under a nitrogen stream, and dissolved in 30 μl of CH3OH:CHCl3 (1:1). The samples were loaded on thin layer chromatography plates that were developed in the lower phase of H2O:ethyl acetate:acetic acid:iso-octane (100:110:20:50). Tritiated phosphatidylbutanol (PtdBut) was measured after the band corresponding to the PtdBut standard was scraped (Avanti Polar Lipids Inc.).

**MMP-9 Secretion and Activity Assay**—Before the assays, the medium was changed and those to be reported elsewhere implicate ARF and PLD in the stimulation of MMP-9 secretion by protein kinase C (PKC).
PLD Mediates PKC Regulation of Golgi Functions

RESULTS

MMP-9 Secretion from HT 1080 Cells in Response to PMA Treatment—To investigate the role of PLD and PA in the induction of MMP-9 secretion from HT 1080 cells, we established a secretion assay that measured MMP-9 activity in medium samples collected after 7.5 h and analyzed for MMP-9 secretion. Secretion was rapidly induced and reached a maximum at 90 min. The response was detectable with 1 nM PMA and maximal at 100 nM PMA (data not shown).

Although the preceding experiments showed that PLD was activated in PMA-treated HT 1080 cells, they provided no evidence that PLD was involved in the secretory pathway. To test this, the cells were treated with various concentrations of a short-chain (dioctanoyl) PA (DOPA). Fig. 3 shows that DOPA induced MMP-9 secretion in a dose-dependent manner with secretion reaching a plateau at 80 μM DOPA. These results demonstrate that PLD activity and the intracellular accumulation of PA are importantly involved in the pathway leading to MMP-9 secretion received further support when 1-propanol was used to block PA production by PLD by virtue of the formation of phosphatidylpropanol through the transphosphatidylation reaction. Cells were first treated with various concentrations of 1-propanol or, for control, 2-propanol and then stimulated with PMA. Medium samples were collected after 7.5 h and analyzed for MMP-9 secretion. Secretion was inhibited by 100 mM 1-propanol but not by the same concentration of 2-propanol (Fig. 4A). At 200 mM, both alcohols were inhibitory, but the effect of 1-propanol was complete. In an additional experiment we checked secretion from cells treated with 133 mM 1- or 2-propanol prior to stimulation with various PMA concentrations. Although MMP-9 secretion was increased by increasing concentrations of PMA in the presence of 2-propanol, 1-propanol almost totally inhibited secretion (Fig. 4B). These results demonstrate that PLD activity and the intracellular accumulation of PA are importantly involved in PMA-dependent MMP-9 secretion.

Prolonged PLD Activity Is Required for MMP-9 Secretion—In the preceding experiments, medium samples for the MMP-9 secretion assay were taken after 7.5 h. This is because the MMP-9 activity in shorter incubations was too low to give reliable quantitative measurements (Fig. 1A). Several reasons could account for this, but one is that prolonged PLD activity is necessary for MMP-9 secretion. To address this possibility, cells were treated with PMA, and 133 mM 1-propanol was

FIG. 1. PMA induces MMP-9 secretion in HT 1080 cells. A, overnight serum-deprived cells were incubated in freshly added DMEM, 0.1% BSA. 100 nM PMA was added at various times to give the indicated durations of exposure to the phorbol ester. Dimethyl sulfoxide (DMSO) solvent was used as the control for PMA. After 7.5 h, medium samples, 5 μl each, were separated on 10% zymogram gels, and MMP-9 activity was assayed as described under “Experimental Procedures.” B, cells were incubated with the indicated concentrations of PMA. After 7.5 h, medium samples were taken and assayed for MMP-9 activity. C, cells were preincubated with or without 5 μM PKC inhibitor Ro 31-8220 for 1 h and then stimulated with dimethyl sulfoxide control or 100 nM PMA for 7.5 h. Medium samples were taken and assayed for MMP-9 activity as described. The data shown for all panels are representative of three independent experiments.

FIG. 2. Activation of PLD in PMA-treated HT 1080 cells. The cells, at their log phase of growth, were serum-deprived and labeled with 1 μCi/ml [3H]myristic acid. Following washing and preincubation with DMEM, 0.1% BSA, 0.3% 1-butanol for 20 min, 100 nM PMA was added, and cells were incubated for the indicated times at 37 °C. Lipids were extracted and analyzed, and the production of [3H]PtdBut was as described under “Experimental Procedures.” [3H]PtdBut values were normalized by dividing the measured counts/min by the counts/min in the total lipid fraction. Data are expressed as the mean ± S.E. of the fold activations in three independent experiments performed in triplicate.
The cells were incubated for 7.5 h after which 5-1-propanol for 45 min. PMA was then introduced to give the indicated concentrations of DOPA. After 7.5 h, 5-night serum-deprived cells were incubated in freshly added DMEM, medium samples were assayed for MMP-9 activity as described under "Experimental Procedures." The data shown are representative of three independent experiments.

Fig. 4. MMP-9 secretion is inhibited by 1-propanol. Overnight serum-deprived cells were incubated with freshly added DMEM, 0.1% BSA, A, the indicated concentrations of either 2-propanol or 1-propanol were then added. After 45 min, 100 nM PMA was added, and cells were incubated for 7.5 h after which 5-μl medium samples were analyzed for MMP-9 secretion. B, the cells were incubated with 133 nM 2-propanol or 1-propanol for 45 min. PMA was then introduced to give the indicated concentrations. The cells were incubated for 7.5 h after which 5-μl medium samples were analyzed for MMP-9 secretion. The data shown are representative of three independent experiments.

added at hourly intervals after the addition of PMA. After 7.5 h, medium samples were collected and analyzed for MMP-9 secretion (Fig. 5). The figure illustrates that the presence of 1-propanol during the first 2–3 h almost fully inhibited MMP-9 secretion. At later times, there was partial suppression of secretion. These data indicate that prolonged formation of PA is important in the action of PMA on MMP-9 secretion.

MMP-9 Secretion Induced by PA Is Not Mediated by Phosphatidic Acid Phosphatase—Although the preceding results implicated PLD and PA formation in the regulation of MMP-9 secretion, they did not prove that PA was the signaling molecule involved. This is because it was possible that PA was converted to a diacylglycerol. Two approaches were used to show that PA rather than diacylglycerol had a role in inducing secretion. Cells were incubated with various concentrations of DOG, and then medium samples were assayed for collagenolytic activity. As is shown in Fig. 6A, DOG had only a minor effect on secretion, even at a concentration higher than that of DOPA. In additional experiments, the secretion of MMP-9 was assayed in cells pretreated with propranolol, an inhibitor of PAP activity (19). Fig. 6B shows that propranolol had no effect on the secretion of MMP-9 from cells treated with either PMA or DOPA.

DISCUSSION

MMP-9 secretion from cancerous cells (15–17) leads to hydrolysis of the extracellular matrix, thus enabling cells to break out of their primary site into the circulation and from there to secondary sites (12–14). MMP-9 secretion is induced by various agonists that differ among various cell lines (15). We have confirmed that in human fibrosarcoma HT 1080 cells, MMP-9 is secreted in response to PMA (17). As expected, the secretary pathway involves the Golgi as revealed by the inhibition of secretion by brefeldin A and other agents that interfere with ARF activation or action.

The most novel finding in the present study is the apparent involvement of PLD in the effect of PMA (PKC) on MMP-9 secretion. Although PMA was shown here to activate PLD in HT 1080 cells as in many other cell types (18), the phorbol ester undoubtedly induced many other changes that could be involved in the secretory response. However, the evidence favors PLD as an important target of PKC in the stimulation of MMP-9 secretion. Importantly, inhibition of PA formation through the addition of the primary alcohol 1-propanol blocked MMP-9 secretion, whereas 2-propanol, which does not participate in transphosphatidylation (20), had much less effect. Furthermore, direct addition of a short-chain PA to the cells was as effective as PMA in stimulating MMP-9 release, supporting the postulated role of PLD.

The present study does not define the cellular site of action of PLD. However, it is very probable that it is the Golgi, because there is much evidence that PLD is involved in protein trafficking involving this organelle (6–11), and exogenous PA can relieve the inhibition of MMP-9 secretion induced by brefeldin A, an inhibitor of Golgi function. In addition, prolonged activation of PLD was required for the effect of PMA (Fig. 5) consistent with its involvement in a relatively slow process. The PLD isoform (PLD1) that has been localized to the Golgi (21) is known to be responsive to both PKC and ARF (22), unlike the PLD2 isoform (21). These observations provide additional support for a site of action at this organelle.

Because PA can be metabolized to other lipids such as diacylglycerol (by PAP) or lysophosphatidic acid (by phospholipase

**Fig. 3.** MMP-9 secretion in response to DOPA treatment. Overnight serum-deprived cells were incubated in freshly added DMEM, 0.1% BSA with the indicated concentrations of DOPA. After 7.5 h, 5-μl media samples were assayed for MMP-9 activity as described under “Experimental Procedures.” The data shown are representative of three independent experiments.

**Fig. 5.** PLD activity is required during the early stages of PMA induction of MMP-9 secretion. Serum-deprived cells were incubated with fresh DMEM, 0.1% BSA. PMA (100 nM) was added at 0 h, and 1-propanol (final concentration of 133 mM) was added at the indicated times thereafter. The medium was sampled after 7.5 h and analyzed for MMP-9 secretion. The data shown are representative of three independent experiments.

**Fig. 6.** Phosphatidic acid phosphatase is not involved in the stimulation of MMP-9 secretion in DOPA-treated cells. Overnight serum-deprived cells were incubated with fresh DMEM, 0.1% BSA. A, the cells were then stimulated by the indicated concentrations of DOG (dried and solubilized by a 30-s sonication in DMEM, 0.1% BSA) or DOPA. B, the cells were incubated with the indicated concentrations of propranolol for 30 min, and then 100 nM PMA or 80 μg/ml DOPA were added. After 7.5 h, 5-μl medium samples were assayed for MMP-9 activity. The data shown are representative of three independent experiments.
A2), the initial results provided no assurance that the observed effects of added PA were because of this lipid per se. However, the possibility that diacylglycerol was the active lipid was rendered unlikely by the observation that the product of PAP action on DOPA (DOG) was largely ineffective. Furthermore, propranolol, an inhibitor of PAP, did not diminish the effect of DOPA. With regard to the possibility that lysophosphatidic acid was the active agent, this seems improbable because the addition of this lipid to HT 1080 cells did not affect MMP-9 secretion.3

Finally, we describe here the characterization of a new model system for the study of Golgi-dependent secretion. In comparison with other methods, the assay of MMP-9 secretion is quick and convenient. It offers an easier and faster way for studying membrane vesiculation and trafficking in response to extracellular signals. Unlike most other methods, it does not require cell permeabilization, virus infection, or any other stressful procedure. Therefore, the cells are in a more physiological state providing information that is as valid as possible in a tissue culture experiment.

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