Phospholipase D (PLD) was shown to be activated by agonists such as platelet-derived growth factor, epidermal growth factor, the chemotactic peptide formyl-Met-Leu-Phe, vasopressin, gonadotropin-releasing hormone, bombesin, and many others (1–3). It is assumed that upon activation of PLD a signal is transmitted downstream via the rise in intracellular phosphatidic acid (PA). When introduced extracellularly, PA was shown to mimic some of the effects of these agonists (4, 5). The mechanism(s) by which PA affects cell function and the identity of its intracellular targets are still not known. Moreover, due to the lack of specific activators or inhibitors of PLD, the causal relationship between PLD-mediated elevation of cellular PA and any specific cell response has not been demonstrated with certainty.

Here we describe the synthesis and characterization of a photolyzable analogue of PA, termed caged PA (cPA), that allows the elevation of the intracellular level of PA upon ultraviolet irradiation. The utility of cPA as a probe was examined in HT 1080 metastatic fibrosarcoma cells. Metastatic spread depends upon the invasive capacity of tumor cells which, in turn, depends on their ability to secrete proteolytic enzymes, such as gelatinase A (MMP-2), that participate in degradation of the basement membrane (6–8). Laminin, an important component of the basal membrane, stimulates MMP-2 secretion (9–11). We have shown recently that extracellularly introduced laminin activates PLD in HT 1080 cells (12). Laminin-induced release of MMP-2 was inhibited by 1-butanol, while addition of exogenous PA or of bacterial PLD into the growth medium mimicked the effect of laminin (12). Thus, HT 1080 cells represent a useful experimental system for studying the second messenger function of PA. We now show that cPA photolysis leads to a rise in intracellular PA and subsequently to secretion of MMP-2 in HT 1080 cells.

**EXPERIMENTAL PROCEDURES**

Materials—All chemicals and solvents used were of reagent grade. Dipalmitoylphosphatidic acid (sodium salt), imidazole, and bovine serum albumin (BSA) were obtained from Sigma. Gelatin was a product of BDH. Hydrazine hydrate, CDCl3, and 2-nitroacetonphenone were from Aldrich. MnO2 (activated grade), MgSO4, and Silica Gel 60 aluminum thin layer chromatography (TLC) plates were purchased from Merck. [32P]ATP and [14C]PA were obtained from DuPont NEN. Diacylglycerol kinase was purchased from Calbiochem. Dulbecco’s modified Eagle’s medium (DMEM), fetal calf serum (FCS), and supplemented antibiotics are products of Biological Industries, Kibbutz Beth HaEmek, Israel.

Cell Lines and Tissue Culture—HT 1080 cells derived from a metastatic lesion of human fibrosarcoma were obtained from the American Type Culture Collection (ATCC) and maintained in DMEM, 10% FCS supplemented with 1000 units/ml penicillin and 100 mg/ml streptomycin. The experiments were carried out with cells which have been serum-deprived by an overnight incubation in DMEM, 0.1% BSA. Before the experiment, the medium was replaced by DMEM without phenol red (DMEM-PR) containing 0.1% BSA to avoid quenching of ultraviolet light by the dye.

Preparation of the Free Acid Form of PA—Phosphatidic acid (sodium salt) was dissolved in chloroform to yield a final concentration of 14 mg/ml. Methanol and 0.2 v HCl were added (chloroform/methanol/HCl ratio of 1:1:0.9, v/v), and the mixture was mixed vigorously. Phase separation was accomplished by centrifugation for 1.5 min at 2000 rpm (Sorval HS-4) at room temperature. The lower phase containing the free acid form of PA was collected, and the pH was adjusted to 4–5 with 10% triethylamine in chloroform.

1 The abbreviations used are: PLD, phospholipase D; BSA, bovine serum albumin; PA, phosphatidic acid; cPA, caged phosphatidic acid; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; MMP-2, matrix metalloproteinase 2.
Synthesis of cPA—The preparation of 1-(2-nitrophenyl)hydrazono-ethane was carried out by dissolving 0.826 g (5 mmol) of 2-nitroacetophenone, 0.562 g (11.2 mmol) of hydrazine hydrate, and 0.32 ml (5 mmol) of glacial acetic acid in 10 ml of ethanol in a 20-ml reflux receptacle. The mixture was refluxed while stirring for 3 h and then was allowed to cool. The ethanol was evaporated in a 25-ml round-bottom flask using a rotary evaporator, and the residue was dissolved in 2 ml of chloroform. Traces of acetic acid were removed by washing the chloroform three times with 1 ml of H2O. Anhydrous MgSO4 (0.5 g) was added, and the mixture was left at room temperature for 5 min, then filtered through cotton, and stored at −20 °C. For the synthesis of 1-(2-nitrophenyl)ldiazooethane, 1.35 ml of chloroform were stirred vigorously with 0.15 ml of 1-(2-nitrophenyl)hydrazonoethane together with 255 mg of MnO2. After 5 min, the mixture was filtered through cotton. This stage and all following stages were performed in subdued light.

1-(2-Nitrophenyl)diazethane was freshly prepared before each synthesis of cPA and used immediately. The final stage in the preparation of 1-(2-nitrophenyl)ethyl ester of PA (namely cPA) was accomplished by stirred PA-free acid solution (10.35 μmol) together with the newly prepared 1-(2-nitrophenyl)diazooethane (155.25 μmol) for 45 min. Then the reaction mixture was applied to an aluminum TLC plate and developed with chloroform, methanol, 25% ammonium hydroxide (65:25:5, v/v). The band of 1-(2-nitrophenyl)ethyl ester of PA was localized by its fluorescence upon illumination of the edges of the plate at a wavelength of 254 nm. The band was developed with chloroform, methanol, 25% ammonium hydroxide (65:25:5, v/v), followed by ultraviolet illumination yielding PA and 2-nitrosoacetophenone. 

NMR Spectroscopy—NMR spectra were obtained on a Bruker AMX 400 NMR spectrometer. The sample was dissolved in DCl, to a final concentration of 2 mg/ml. Peak assignment was aided by comparison of the precursors and the cPA. Data were analyzed on a Personal Iris Workstation (Siemens Graphics).

Loading of Cells with cPA and Its Photolysis—cPA was solubilized by a 30-min sonication in a bath sonicator in DMEM-PR, 0.1% BSA. The solubilized cPA was introduced into the medium of overnight serum-deprived HT 1080 cells in a final concentration of 16 μM for 1 h, unless otherwise indicated. At the end of the incubation, excess cPA was removed by washing the cells twice with DMEM-PR, 0.1% BSA. Photolysis was carried out by illuminating the culture plates from a distance of 10 cm with 365 nm wavelength lamps (315 and 365 nm wavelength). Determination of Cellular PA Mass—Lipids were extracted from cultures of resting HT 1080 cells (5) and separated by two-dimensional TLC using chloroform/methanol/NH4OH (65:25:5) in the first dimension and chloroform/acetonitrile/methanol/acetic acid/water (30:40:10:10:5) in the second dimension. Standard curves of PA were run in parallel with PA mass was determined by Coomasie Blue staining essentially according to Kaszkin et al. (19).

Determination of MMP-2 Activity—MMP-2 activity was determined as described previously (12). Results are expressed as the measured intensity relative to that of control.

RESULTS AND DISCUSSION

The synthesis of cPA is based on alkylation of the weakly ionized phosphate group of PA by 1-(2-nitrophenyl)diazooethane (Fig. 1A). In the first step, 1-(2-nitrophenyl)hydrazonoethane is synthesized from 2-nitroacetophenone by refluxing it with hydrazine hydrate in the presence of protons donated by glacial acetic acid. In the second step, 1-(2-nitrophenyl)hydrazonoethane is oxidized by MnO2 to yield 1-(2-nitrophenyl)diazethane which was later allowed to react with PA resulting in the desired 1-(2-nitrophenyl)ethyl ester of PA. In general, the preparation of cPA is similar to the preparation of other caged compounds (15–17). The main difference is in the last stage of the preparation which was accomplished in a single phase since here both reactants are hydrophobic and soluble in chloroform. As demonstrated in Fig. 1B, the 1-(2-nitrophenyl)ethyl moiety, which in cPA is linked to the phosphate group of the PA, is removable upon ultraviolet illumination at a wavelength of 300–400 nm.

The expected structure of cPA and its photolytic products was verified by comparing the proton NMR spectra of cPA, PA, and photolyzed cPA (Fig. 2). The spectrum of cPA clearly shows the additional peaks contributed by the alkylation group (Fig. 2A). In particular, note the aromatic signals of the nitrophenyl group and the distinct appearance of the doublet at 1.7 ppm and the additional peaks contributed by the alkylating group (Fig. 2A). Those peaks disappeared upon ultraviolet irradiation, and the spectrum of the photolyzed cPA is nearly identical with that of PA (Fig. 2B).

Next we have measured the incorporation of cPA into HT 1080 cells by introducing [32P]cPA into the growth medium. The uptake of cPA was time-dependent, reaching a plateau after 120 min (Fig. 3A). In all additional experiments, loading was accomplished by incubating the cells with cPA for 1 h, after which uptake was 1327 pmol/S × 105 cells, representing 8.3% of the cPA introduced into the medium. In vivo photolysis was demonstrated in [32P]cPA-loaded cells that were irradiated for various times (Fig. 3B). There was a direct relationship between the time of irradiation and [32P]cPA accumulation. The results demonstrate that cPA is incorporated into the cells in a time-dependent manner. It is also shown that the increase in PA is correlated to the length of irradiation. The uptake of cPA was studied also in NIH 3T3 and Swiss 3T3 cells. These cells were found to be much more sensitive to an apparently cytotoxic effect of cPA at the concentrations used.

MMP-2 is a key determinant of the metastatic potential of...
tumor cells. MMP-2 production in HT 1080 is stimulated by laminin (9–11). We have previously demonstrated that in HT 1080 cells laminin activates PLD (12). We hypothesized that the rise in PA level elicits MMP-2 release and that cPA photolysis would mimic this effect. The effect of cPA photolysis on MMP-2 release was compared to that of exogenously added PA (Fig. 4). cPA had no effect on MMP-2 release in nonilluminated cells (B) or in cells illuminated before its addition (A). Photolysis of cPA in cPA-loaded cells (C) caused a 2.1-fold increase in the activity of MMP-2 measured in the growth medium. In comparison, exogenous PA elevated MMP-2 release by 66% in illuminated cells. Incubation of HT 1080 cells with the byproduct of cPA photolysis, 2-nitrosoacetophenone, had no effect on MMP-2 release, even when incubated with the cells for 3 h at very high concentrations (100 μM) (data not shown). It may thus be concluded that cPA photolysis mimics the effect of laminin in these cells by elevating the level of PA.

The optimal duration of irradiation was tested by illuminating cPA-loaded cells for various times and measuring release of MMP-2 (Table I). It is illustrated that short photolysis was more effective than long photolysis. As demonstrated earlier (Fig. 1B), efficient in vitro photolysis of cPA required irradiation longer than 60 s. However, exposing cells to ultraviolet irradiation for more than 20 s dramatically reduced their viability.3 On the other hand, as demonstrated here, a 5-s illumination was sufficient to photolyze enough cPA to mimic the effect of laminin on MMP-2 secretion. The reduction in the effectiveness of cPA photolysis on MMP-2 release, found with illumination periods longer than 5 s, might be explained by the greater damage caused to the cells by the ultraviolet light.3

To identify cellular processes that are directly modulated by PA, it is necessary to be able to experimentally cause very rapid changes in cellular PA concentrations. The introduction of caged PA derivatives into cultured cells is a novel approach that allows experimental elevation of intracellular PA. Light-induced generation of PA mimics signal-dependent activation of PLD, while physiological receptor, transducer, and effector
Fig. 4. Induction of MMP-2 release upon illumination of cPA
loaded cells. DMEM-PR, 0.1% BSA (bars 1, 3, 4, and 6) or DMEM-PR, 0.1% BSA containing 16 \( \mu \)g cPA (bars 2, 5, and 7) were added to nonilluminated cells (B and C) or to 5-s illuminated cells (A). After 1 h, the medium was removed and all plates were washed twice with DMEM-PR, 0.1% BSA. cPA photolysis was accomplished by ultraviolet illumination for 5 s (C, bar 7) while control cells were not illuminated (bars 2 and 5). PA was added to a final concentration of 50 \( \mu \)g/ml (bars 3 and 6). After a 24-h incubation at 37 \( ^\circ \)C, 75 \( \mu \)l of medium samples were removed and MMP-2 activity was tested as described under “Experimental Procedures.” The results are expressed as the activity relative to control (no treatment, bar 4).

Table I

Dependence of MMP-2 release upon length of exposure to ultraviolet light.

| Time of Illumination | MMP-2 Release |
|----------------------|---------------|
| 0                    | 1.0           |
| 5                    | 2.25 ± 0.04   |
| 10                   | 1.96 ± 0.13   |
| 20                   | 1.84 ± 0.14   |
| 30                   | 1.13 ± 0.13   |

mechanisms are bypassed. This approach offers the possibility of eliciting very rapid changes in membrane levels of PA in the absence of parallel, hormone-induced activation of other signaling pathways.

In the present study it was demonstrated that cPA photolysis stimulates MMP-2 release whereas nonphotolyzed cPA had no effect, and that the effect of photolyzed cPA is about twice the effect of extracellularly added PA. The effect of cPA photolysis is likely due to the elevation of intracellular PA. Illumination for 5 s caused photolysis of 1.8% of the cPA (24 pmol/5 \( \times 10^5 \) cells). The mass of PA in resting HT 1080 cells was determined by a two-dimensional TLC/Coamassie Blue staining procedure and found to be 31 pmol/5 \( \times 10^5 \) cells. Therefore, it seems that even a relatively small elevation in PA mass is sufficient for eliciting a significant cellular response. In other studies it has been shown that agonist activation caused elevations of PA mass that were higher by approximately one order of magnitude (18–22). These greater elevations were obtained with a high concentration of agonists, representing a maximal or near-maximal activation of PLD and other pathways. However, it does not necessarily represent the physiological change needed for PA to exert its effect. Another possible explanation is that cPA photolysis forms microdomains of PA concentration which are sufficient for causing its biological effects.

The utilization of cPA offers a number of possibilities for studying the metabolism and action of PA in cells and cell-free preparations. Caged PA derivatives with either radioisotopically labeled or fluorescently labeled phosphatidyl moieties of different fatty acyl chain length could be employed to follow the uptake, transport, and metabolism of cPA and PA, before and after photolysis, respectively. This could provide hitherto unobtainable information regarding the metabolic fate of the PA produced by the signal-activated PLD. In addition, cPA photolysis could potentially be exploited to identify immediate biochemical responses that are directly regulated by PA, e.g., changes in activity of specific enzymes, protein phosphorylation patterns, or ionic currents.

At present, the utility of cPA is somewhat limited by the low efficiency of the loading step. It may be expected (23, 24) that the exchange of cPA with cells (and hence the efficiency of its uptake) will be improved by using short chain analogs of cPA. Another factor that limits the usefulness of cPA is its apparent toxicity in some cell lines. The mechanism of this effect is not clear. Certain cell types are more sensitive to the “cage” moiety utilized in cPA (15, 16). One way to overcome this problem will be to use photosensitive blocking groups other than the 1-(2-nitrophenyl)ethyl moiety that was employed here.

We have previously shown that laminin stimulates PLD activity in HT 1080 cells (12). A causal relationship between PLD activation and the subsequent laminin-induced release of MMP-2 was suggested by the fact that the effect of laminin was inhibited by 1-butanol, an alternative substrate of PLD that attenuates PA production by shunting phosphatidyl moieties from PA into phosphatidylbutanol. Furthermore, laminin-induced release of MMP-2 could be mimicked by treatment of the cells with an exogenous (bacterial) PLD. In the present study it is shown that light-induced generation of PA by photolysis of cPA also can mimic the action of laminin on MMP-2. Collectively, these data strongly suggest a pivotal role for PLD and PA in the signaling cascade of laminin-induced MMP-2 release and, consequently, in tumor cell invasiveness and metastasis. The photolysis of cPA is likely to be useful in elucidating the downstream biochemical events that follow PLD activation in these cells, causing changes in gene expression and culminating in malignant dissemination.

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Release of Gelatinase A (Matrix Metalloproteinase 2) Induced by Photolysis of Caged Phosphatidic Acid in HT 1080 Metastatic Fibrosarcoma Cells
Ben-Tsion Williger, Reuven Reich, Michal Neeman, Tuvia Bercovici and Mordechai Liscovitch

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