Phospholipase C–mediated Hydrolysis of Phosphatidylcholine Is Activated by *cis*-Diamminedichloroplatinum(II)

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Abstract

We have investigated the effect of *cis*-diamminedichloroplatinum(II) (CDDP) on signal transduction pathways. CDDP treatment did not cause any change in the binding of [^{3}H]-phorbol dibutyrate to PC-9 (human lung adenocarcinoma cell line) cells, a measure of protein kinase C activation. However, 2-h CDDP treatment (20 μg/ml) caused ~ 200% increase in 1,2-*sn*-diacylglycerol (DAG) production and ~ 50% decrease in inositol 1,4,5-triphosphate production. To explore the different source of DAG, we analyzed phospholipids labeled with [^{14}C]choline by TLC and revealed that [^{14}C]choline-labeled phosphatidylcholine (PC) was decreased to 50% by CDDP treatment. This suggested that PC turnover was increased by CDDP-treatment. PC-specific phospholipase C (PC-PLC) activity was increased to 2.5-fold (2.58±0.28 nmol/mg protein per min) by 2 h CDDP (20 μg/ml) treatment compared with control (1.05±0.24 nmol/mg protein per min). Treatment of CDDP also stimulated PC-PLC in the crude membrane extract from PC-9 cells. CDDP had no effect on the activities of phospholipase A2 and D. *Trans*-DDP, which has far less cytotoxicity than its stereoisomer, CDDP, did not cause any change in PC-PLC activity. A significant inhibition of DNA synthesis (< 80%) occurred 4 h after 2 h CDDP (20 μg/ml) treatment. These results demonstrated that CDDP-induced PC-PLC activation was an early event in CDDP-induced cytotoxicity and suggested that the effects of CDDP on signal transduction pathways had an important role in CDDP-induced cytotoxicity. (*J. Clin. Invest.* 1992. 89:1622–1628.) Key words: *cis*-diamminedichloroplatinum(II) • phosphatidylcholine • phospholipase C

Introduction

cis-diamminedichloroplatinum(II) (CDDP)[1] is a key anticancer agent for the treatment of solid tumors (1). Phorbol esters, such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA), have various biological effects on a variety of cellular functions (2, 3). Several recent studies have shown that TPA could modulate CDDP-induced cytotoxicity (4–6). Hofmann et al. (4) have observed sensitization of Walker rat carcinoma cells to CDDP by long-term (48-h) exposure to TPA and have postulated that the sensitization effect of TPA resulted from the inhibition or downregulation of protein kinase C (PKC). Basu et al. (5) have also observed that long-term (24-h) pretreatment with TPA sensitized HeLa cells to CDDP, but they have shown that the downregulation of PKC could not explain the sensitizing effect of TPA and postulated that activation of PKC was necessary for sensitization to CDDP. Isonishi et al. (6) have reported that short-term (1-h) TPA exposure could sensitize 2,008 ovarian carcinoma cells to CDDP. Although they did not measure the actual PKC activity, they suggested that CDDP sensitivity could be modulated by PKC.

We have recently reported that CDDP-resistant human lung cancer cell line was cross-resistant to the growth-inhibitory effect of TPA (7). Considering that TPA modulated CDDP-induced cytotoxicity and that CDDP-resistant cells showed cross-resistance to TPA, we can speculate that TPA and CDDP have a somewhat common mechanism of action in their growth-inhibitory effect and cytotoxicity.

The effects of TPA appear to be mediated largely through signal transduction pathways involving PKC activation (2, 3). Recent evidence suggests that TPA acts on phosphatidylinositol (PI)-specific phospholipase C (PI-PLC) (8–12) and phosphatidylcholine (PC)-specific phospholipase C (PC-PLC) (13–23), both of which are considered to be important enzymes in signal transduction pathways. However, there have been few reports describing the effect of CDDP on signal transduction pathways; the reports have focused only on PKC activity (24).

For this report we investigated the effect of CDDP on signal transduction pathways and demonstrated that CDDP has no effect on PKC activity and that CDDP activates PC-PLC. This PC-PLC activation occurred before CDDP-induced inhibition of DNA synthesis. *Trans*-diamminedichloroplatinum(II) (*trans*-DDP) did not cause PC-PLC activation. Therefore, the effect of CDDP on signal transduction pathways might have an important role in CDDP-induced cytotoxicity.

Methods

Chemicals. CDDP was obtained from Bristol-Myers Squibb Japan (Tokyo, Japan). RPMI 1640 and calcium- and magnesium-free PBS

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1. *Abbreviations used in this paper:* CDDP, *cis*-diamminedichloroplatinum(II); DAG, 1,2-*sn*-diacylglycerol; dH_2O, distilled water; G proteins, GTP-binding proteins; IP_3, inositol 1,4,5-trisphosphate; LPC, lysophosphatidylcholine; PA, phosphatidic acid; PBt_2, phorbol dibutylate; PC, phosphatidylcholine; PC-PLC, PC-specific phospholipase C; PI, phosphatidylinositol; PI-PLC, PI-specific phospholipase C; PKC, protein kinase C; PLA_2, phospholipase A_2; PLD, phospholipase D; SM, sphyngomyelin; Thd, thymidine; *trans*-DDP, *trans*-diamminedichloroplatinum(II); TPA, 12-*O*-tetradecanoyl phorbol 13-acetate.

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were purchased from Nissui Pharmaceutical Co. (Tokyo, Japan). [γ-^{32}P]ATP; [^{14}C]choline; PC, 1-stearoyl-2-[methyl-^{14}C]arachidonyl ([^{14}C]PC), and [^{3}H]thymidine ([^{3}H]Thd) were purchased from Amersham Japan (Tokyo, Japan). Other drugs and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) if not otherwise mentioned.

Cell cultures. PC-9 is a human non-small cell lung cancer cell line (25). Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified 5% CO_2 atmosphere at 37°C.

Assay for cellular 1,2-sn-diacylglycerol (DAG). At various time periods after the addition of 20 μg/ml CDDP, lipids of PC-9 cells (2×10^5 cells) were extracted with chloroform/methanol (2:1) (vol/vol). Then, we added 1.25 vol of chloroform and 1.25 vol of 0.2 M KCl-5 mM EDTA solution. After centrifugation at 1,000 *g* for 10 min at 4°C, lipids in organic phase were extracted by the modified method of Bligh and Dyer (26). Samples in the organic phase were dried under N_2 gas, and DAG mass was assayed according to the method of Preiss et al. (27). The assay was linear with respect to DAG mass from 0.2 to 5 nmol.

Assay for inositol 1,4,5-trisphosphate (IP_3). At various time periods after the addition of 20 μg/ml CDDP, 1 ml of PC-9 cell suspension (2×10^5 cells/ml) was mixed with 200 μl ice-cold 20% perchloric acid and kept on ice for 20 min. Proteins were sedimented by centrifugation at 2,000 *g* for 15 min at 4°C. Supernatants were transferred to the new tubes and were neutralized to pH 7.5 by 1 M KOH and kept on ice for 1 h. Then we added 4 ml distilled water (dH_2O) to the neutralized supernatants. This solution was applied to the minicolumn (Amprep, Amersham) at a flow rate of 3 ml/min. The column was washed once with 5 ml of dH_2O and once with 5 ml of 0.1 M $KHCO_3$ at the same flow rate. The IP_3 fraction was eluted with 5 ml of 0.17 M $KHCO_3$ and was collected. This 100 μl of IP_3 fraction was measured by a competitive binding assay (Amersham IP_3 assay kit).

Analysis of phospholipid labeled with [^{14}C]choline. Cells (2×10^5) were labeled with 2 μCi of [^{14}C]choline (sp act 50–60 mCi/mmol) for 48 h. The last 24 h of labeling was performed in serum-free medium. Labeled cells were washed once with warmed PBS and were exposed to various concentrations of CDDP for 2 h. Reactions were terminated by removing the supernatants and the cells were washed three times with cold medium containing unlabeled 1 mM choline. Then we added 2 ml of ice-cold methanol and transferred the cells to glass tubes after a 10-min incubation at 4°C. The washed culture dish was rinsed twice with 1 ml of ice-cold methanol and we added this solution to the glass tubes mentioned above. We then added 2 ml of chloroform and left the extracts for 1 h at 4°C. The tubes were then centrifuged at 400 *g* for 10 min. Organic phases were dried under N_2 gas and lipids were fractionated by TLC using the following solvent systems. For the fractionation of different phospholipids, chloroform/methanol/concentrated ammonia (65:25:4) (vol/vol/vol) was used in the first dimension and chloroform/acetone/methanol/acetic acid/water (30:40:10:10:5) (vol/vol/vol/vol/vol) was used in the second dimension. The spot corresponding to each lipid, located by autoradiography, was scraped off the plate; and the radioactivity of each lipid was measured in a liquid scintillation counter.

Preparation of membrane fraction. Subconfluent cells were harvested and washed twice with ice-cold buffer 1 (PBS containing 1 mM EDTA [pH 7.3]). Collected cells were resuspended in buffer 2 (2 mM Hepes, 154 mM NaCl, 1 mM EDTA, pH 7.4) at 6×10^6 cells/ml. After freezing and thawing twice, the cell suspension was sonicated in a bath sonicator for 30 s. Before ultracentrifugation, an aliquot of fresh sonicate was centrifuged at 180 *g* for 10 min at 4°C. The supernatant was then centrifuged at 100,000 *g* for 90 min at 4°C (Ultracentrifuge TL-100 with a fixed-angle rotor TL-45, Beckman Instruments, Fullerton, CA). After ultracentrifugation the pellet was resuspended in buffer 2. Membrane fractions were immediately frozen at −80°C until use. Protein content was measured by the method of Lowry et al. (28).

Analysis of PC hydrolysis in PC-9 cells. [^{14}C]PC (sp act 56 mCi/mmol) was dried under N_2 gas and then was stored in chloroform at −20°C until use. At the time of the experiments, stocked 22.4 μCi [^{14}C]PC was suspended in 100 μl of dH_2O and was sonicated in a bath sonicator for 2 min at room temperature. 50 μg membrane fraction proteins were incubated with 100 μl of reaction buffer, 100 μl of [^{14}C]PC solution and 100 μl of 6 mM $CaCl_2$ for 1 h at 37°C. Reaction buffer consisted of 100 mM Hepes and 100 mM sodium acetate (pH 7.5). Parallel reactions, in which 5 U of phospholipase A_2 (PLA_2) or 10 U of PC-PLC (Seikagaku Kogyo Co., Tokyo, Japan) were included instead of membrane fraction proteins, were performed as control experiments. Reactions were terminated by the addition of 1 ml of chloroform/methanol (2:1) (vol/vol), which contained 36 mM HCl. All measurements were performed in triplicate. Then nonradiolabeled lipid mixture (60 nmol each of PC, lysophosphatidylcholine [LPC], oleic acid, DAG, and sphingomyelin [SM]) was added just before lipid extraction for visualization of PC, LPC and DAG on TLC plates. The solutions were mixed and incubated for 1 h at 4°C. After incubation, phase separation was facilitated by centrifugation at 200 *g* for 5 min. The chloroform phase was transferred to a new glass tube. The residual aqueous phase was extracted again with 0.8 ml of chloroform and combined with the former chloroform phase. The pooled chloroform phases were dried under N_2 gas and dissolved in 20 μl of chloroform/methanol (2:1) (vol/vol) and then applied to the silica gel F254 TLC. For the fractionation of [^{14}C]DAG, diethyl ether/benzene/ethanol/triethylamine (40:50:2:1) (vol/vol/vol/vol) was used as the first-dimension solvent system. Chloroform/methanol/acetic acid (85:14:1) (vol/vol/vol) was used as the second-dimension solvent system. For the fractionation of [^{14}C]LPC and [^{14}C]phosphatidic acid ([^{14}C]PA), the plates were developed in chloroform/methanol/concentrated ammonia (65:35:5) (vol/vol/vol). LPC (R_f = 0.10), PA (R_f = 0.05), and SM (R_f = 0.17) were completely separated from PC (R_f = 0.39) when the distance of solvent front from origin was 17 cm.

After drying, TLC plates were exposed to iodine vapor for 1 h. The spots corresponding to the lipid standards were marked and were scraped off the plates. Then the radioactivity was counted by a liquid scintillation counter. About 95% of the radioactivity could be recovered. PLA_2 activity was quantitated by the release of [^{14}C]LPC from [^{14}C]PC. PC-PLC activity was quantitated by the release of [^{14}C]DAG from [^{14}C]PC. Depending on the substrate used and the activity being assayed, the product spots usually gave 2,000–20,000 cpm counts.

Analysis of PC-PLC and PLA_2 activities in CDDP-treated cells. PC-9 cells were treated with various concentrations of CDDP for 2 h. Cells were harvested and the membrane extraction was performed according to the methods described in "Preparation of membrane fraction." The activities of PC-PLC and PLA_2 in the extracts were analyzed by the same methods described in "Analysis of PC hydrolysis in untreated cells."

Analysis of the effects of CDDP and trans-DDP on PC-PLC and PLA_2 in the crude cell extracts. At the time of the experiments, stocked 22.4 μCi [^{14}C]PC was suspended in 100 μl dH_2O and was sonicated in a bath sonicator for 2 min at room temperature. 50-μg membrane fraction proteins were incubated with 50 μl of CDDP or 120 μg/ml *trans*-DDP, which gave a final concentration of 20 μg/ml; 100 μl of reaction buffer; 100 μl of [^{14}C]PC solution; and 50 μl of 12 mM $CaCl_2$ for 2 h at 37°C. The content of the reaction buffer was described in "Analysis of PC hydrolysis in untreated cells." After incubation we performed the same procedure described in "Analysis of PC hydrolysis in untreated cells."

DNA synthesis. 2×10^6 cells were treated with 20 μg/ml of CDDP or PBS as the control for 2 h. After incubation CDDP was removed and then the cells were incubated in a humidified atmosphere of 5% CO_2-95% air in the complete medium for 0–18 h. At each time point, cells were resuspended in 1 ml of fresh complete medium containing 2 μCi/ml of [^{3}H]Thd (sp act 6.7 Ci/mmol) and incubated for 30 min to produce radiolabeled DNA. The cells were then collected to a 15-ml centrifuge tube and were rinsed twice with ice-cold PBS. We added 10 μl of horse serum as a carrier and 5 ml of 10% ice-cold TCA and mixed well. This mixed solution was incubated on ice for 15 min, and the precipitate was collected by centrifugation at 1,500 *g* for 10 min at 4°C. 200 μl

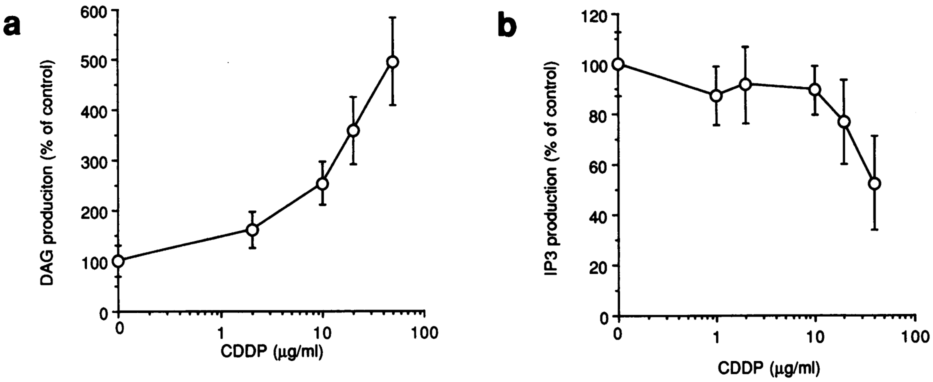

Figure 1. Dose response of the effect of CDDP on DAG production, IP_3 production, and $[^3H]PBt_2$ binding to PC-9 cells. (*a*) PC-9 cells were treated for 2 h with various concentrations of CDDP. The DAG mass in the organic phase of an extract of the cells was measured by the use of *Escherichia coli* DAG kinase. DAG mass was obtained from the standard curve and the results (triplicate determination in two experiments) are expressed as DAG in CDDP-treated cells as a percentage of DAG in control PC-9 cells, which contain 1.5 nmol (900 cpm) of DAG per 2×10^6 cells. (*b*) PC-9 cells were treated for 2 h with various concentrations of CDDP. IP_3 production was measured by the use of an IP_3 assay kit. The amount of IP_3 was obtained from the standard curve and the results (triplicate determination in two experiments) are expressed as IP_3 in treated cells as a percentage of IP_3 in control PC-9 cells (which contains 1,630 cpm).

of folic acid (Wako Pure Chemical Co., Osaka, Japan) was added to solubilize the pellets. The radioactivity was measured in a liquid scintillation counter.

Results

Effect of CDDP on PKC and PI-hydrolysis. To examine the effect of CDDP on signal transduction pathways, we initially determined the dose-dependent effect of 2 h CDDP treatment on DAG production, IP_3 production, and phorbol dibutylate (PBt_2) binding to PC-9 cells. The activation of PKC has been correlated with its translocation from the cytosol to cellular membranes and a subsequent increase in the binding of $[^3H]$-PBt_2 to intact cells (29, 30). Therefore, PBt_2 binding reflects PKC activity. DAG production was stimulated in a dose-dependent manner by 2 h CDDP treatment (Fig. 1 *a*). However, IP_3 production was inhibited by higher concentrations (> 20 μg/ml) of CDDP treatment (Fig. 1 *b*) and we could not observe any change of $[^3H]PBt_2$ binding to the cells after various concentrations of CDDP treatment for 2 h (data not shown). The PKC content was also not affected by various concentrations of CDDP treatment (data not shown).

The physiological activation of PKC generally results from acute increase in cellular DAG content. In the following experiments, we examined DAG production, IP_3 production, and PBt_2 binding to the cells at various times within 2 h after the addition of CDDP (20 μg/ml). By the use of a colony formation assay (2 h CDDP exposure), CDDP concentration of 20 μg/ml killed ~ 90% of the cells at the time of colony counting (day 10) (unpublished data). However, 2 h after the addition of 20 μg/ml CDDP, we could not observe any decrease in cell numbers or viability as counted by trypan blue staining. CDDP treatment caused an increase of DAG production 30 min after the addition of CDDP (Fig. 2 *a*). DAG production doubled after 2 h. However, unexpectedly, there no increase of PBt_2 binding to the PC-9 cells occurred within 2 h (data not shown) and a significant inhibition of IP_3 production (Fig. 2 *b*) was observed. The hydrolysis of phosphatidylinositol 4,5-bisphosphate by PI-PLC is an important source of DAG and IP_3, but it is now known that PC can also be hydrolyzed by PC-PLC to yield DAG. To explore the different source of DAG, the following experiment was carried out.

CDDP treatment increased PC turnover. To examine the effect of CDDP addition to quiescent PC-9 cells on PC-PLC–

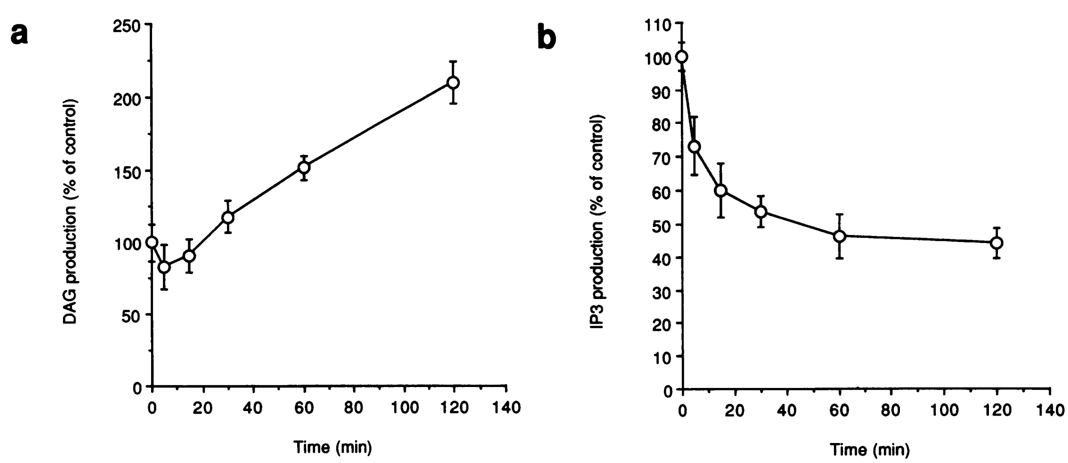

Figure 2. Time course of the effect of CDDP on DAG production, IP_3 production, and PBt_2 binding to PC-9 cells. (*a*) At various times after the addition of 20 μg/ml CDDP, the DAG mass in the organic phase of an extract of PC-9 cells was measured by the use of *E. coli* DAG kinase. DAG mass was obtained from the standard curve and the results (triplicate determination in two experiments) are expressed as DAG in CDDP treated cells as percentage of DAG in control PC-9 cells, which contain 1.5 nmol (900 cpm) of DAG per 2×10^6 cells. (*b*) At various times after the addition of 20 μg/ml of CDDP, IP_3 production was measured by the use of an IP_3 assay kit. The amount of IP_3 was obtained from the standard curve and the results (triplicate determination in two experiments) are expressed as IP_3 in treated cells as a percentage of IP_3 in control PC-9 cells (which contains 1,630 cpm).

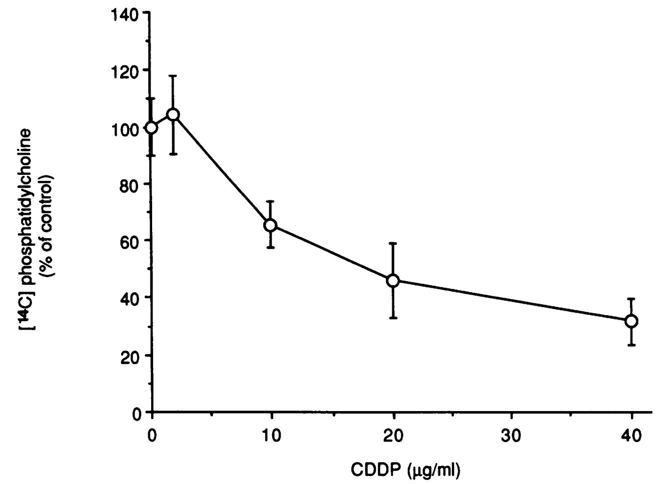

Figure 3. Dose response of the effect of CDDP on PC labeled with [^{14}C]choline. PC-9 cells (2×10^5 cells) were preincubated for 48 h with 2 μCi of [^{14}C]choline. Cells were exposed to various concentrations of CDDP for 2 h. After CDDP treatment, labeled lipids were extracted and were analyzed by TLC. Basal [^{14}C]choline incorporation into PC is 72,000 cpm per 2×10^5 cells.

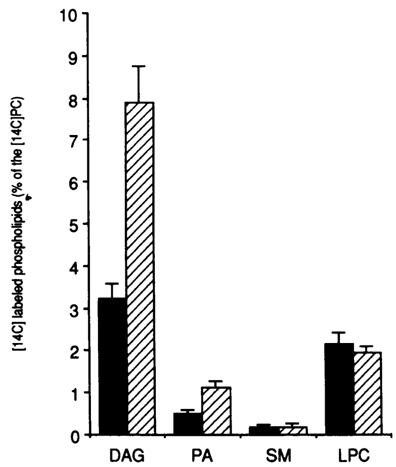

Figure 5. Hydrolyzed products of PC in intact PC-9 cells and CDDP-treated PC-9 cells. 50 μg of membrane fraction proteins extracted from PC-9 cells, either untreated (*black bar*) or treated (*shadow bar*) by 20 μg/ml of CDDP, were incubated for 1 h with 22.4 μCi of [^{14}C]PC in the appropriate reaction buffer. ^{14}C-labeled lipids were extracted and were analyzed by TLC.

mediated hydrolysis of PC, PC-9 cells were labeled with [^{14}C]-choline for 48 h and then treated by various concentrations of CDDP for 2 h. The last 24 h labeling was performed in a serum-free medium. After 48 h labeling, the levels of ^{14}C-labeled PC became saturated (data not shown). Results shown in Fig. 3 indicate that the level of ^{14}C-labeled PC was decreased in a dose-dependent manner after 2 h CDDP treatment. The decrease was observed above 2 μg/ml of CDDP concentrations and in fact the change of ^{14}C-labeled PC was inversely correlated with the change of DAG production. Furthermore, the level of ^{14}C-labeled PC was decreased in a time-dependent manner when the cells were treated with 20 μg/ml of CDDP for 2 h. After 2 h, the level of ^{14}C-labeled PC was 50% of that of control cells (data not shown). Considering the results that CDDP increases PC turnover, we then examined whether PC hydrolysis was affected by CDDP treatment in the following experiments.

CDDP treatment increased PC-PLC activity, but not PLA_2 activity. Formation of PA and DAG by stimulated cells could occur by several distinct pathways (Fig. 4). PC can be hydrolyzed by PC-PLC to yield DAG described above. The resultant DAG is then phosphorylated by DAG kinase to PA (31). PA is also formed by direct action of phospholipase D (PLD) on PC (32–39). SM is also formed by PLD. PA, thus, is formed from PC and DAG by PLD and DAG kinase. On the other hand, PLA_2 formed LPC and arachidonic acids from PC. We compared each production of DAG, LPC, PA, and SM before and after CDDP treatment. As shown in Fig. 5, PA and SM productions were much lower than those of DAG and LPC. And after CDDP treatment we observed no change of SM production

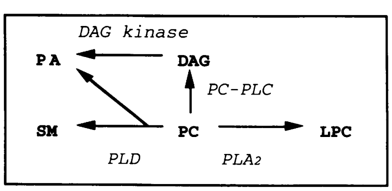

Figure 4. Several pathways from PC by lipases.

and slight increase in PA production. Considering the fact that PA is both a PLD-mediated hydrolyzed product of PC and also a DAG kinase–mediated phosphorylated product of DAG, it appears to be unlikely that PLD activity was affected by CDDP treatment. In addition, the fact that the ratio of [^{14}C]PA to [^{14}C]DAG remained constant before and after CDDP treatment suggested that DAG kinase was also not affected by CDDP treatment.

We then examined whether PC-PLC and PLA_2 activities were affected by CDDP treatment. Membrane fractions from the PC-9 cells with or without 2 h CDDP (20 μg/ml) treatment were used for the measurement of each enzyme activity. PC-PLC activity was calculated from [^{14}C]DAG production. PLA_2 activity was calculated from [^{14}C]LPC production. Without CDDP treatment, PC-PLC activity was 1.05±0.24 (nmol/mg protein per min) and PLA_2 activity was 0.55±0.18 (nmol/mg protein per min). After 20 μg/ml of CDDP treatment for 2 h, PC-PLC activity increased ~ 2.5-fold (2.58±0.28 nmol/mg protein per min), but PLA_2 activity (0.50±0.17 nmol/mg protein per min) was almost same as the control experiment. We also examined these enzyme activities in homogenates of whole cells. We could find the lack of change in LPC in whole cells in the same treatment condition. On the other hand, the activation of PC-PLC activity by CDDP was also observed in whole cells. These results support the activation of PC-PLC and the lack of activation of PLA_2 in membranes. These findings are consistent with the findings demonstrating the increased DAG production and increased PC turnover in CDDP-treated cells.

To examine whether CDDP-induced PC-PLC activation was related to CDDP-induced cytotoxicity, we examined the in vitro effect of CDDP and *trans*-DDP on PC-PLC activity, respectively. *Trans*-DDP has far less cytotoxic ability than its stereoisomer, CDDP. Results in Fig. 6 *a* clearly indicate that CDDP caused an increase in PC-PLC activity, but *trans*-DDP did not cause any change of PC-PLC activity. Moreover, in agreement with the results obtained from in vivo experiments, results in Fig. 6 *b* demonstrated that neither CDDP nor *trans*-DDP affected PLA_2 activity. These results suggested that CDDP-induced PC-PLC activation is related to CDDP-induced cytotoxicity.

Inhibition of DNA synthesis after 2 h CDDP treatment. DNA is the accepted target for CDDP cytotoxicity, but recent evidence shed doubt on DNA synthesis as the critical process

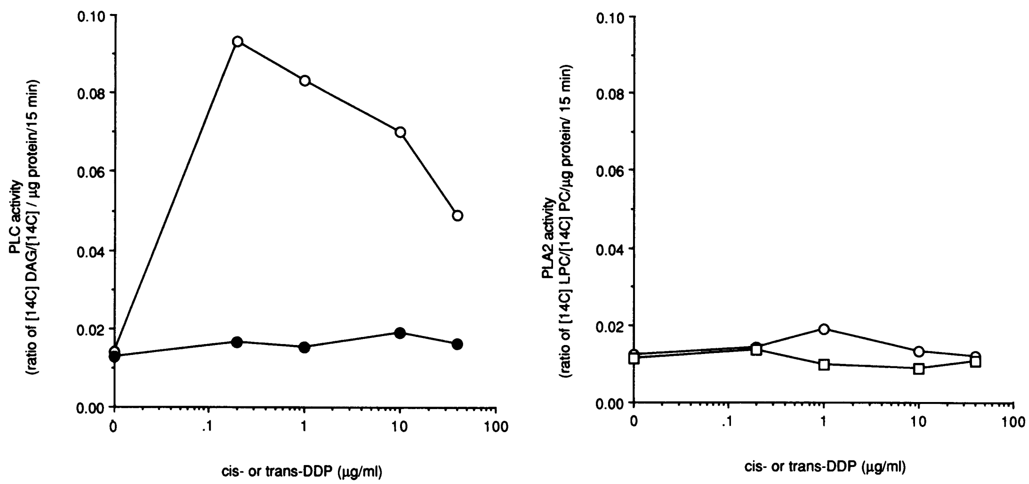

Figure 6. Dose response of the effect of CDDP and *trans*-DDP on PC-PLC and PLA_2 in the membrane fraction of PC-9 cells. Membrane fraction was extracted from intact PC-9 cells. 50-μg membrane fraction proteins were incubated for 1 h with various concentrations of CDDP (○) or *trans*-DDP (●) and 22.4 μCi of [^{14}C]PC in the appropriate reaction buffer. PC-PLC (*a*) and PLA_2 (*b*) activities were measured using [^{14}C]PC and [^{14}C]LPC as a parental compound, respectively. Basal PC level was 18,000–20,000 cpm. PC-PLC activity was expressed as the percentage of [^{14}C]DAG per parental [^{14}C]PC. PLA_2 activity was expressed as the percentage of [^{14}C]LPC per parental [^{14}C]PC.

(40). Therefore, we determined the sequence of events (PC-PLC activation and inhibition of DNA synthesis) occurring in cells after CDDP treatment. 2 h CDDP treatment caused 2.5-fold PC-PLC activation, and a significant inhibition of DNA synthesis occurred 4 h after CDDP (20 μg/ml) treatment (Fig. 7), with no change of Thd transport across the cell membrane (data not shown). It appeared that CDDP-induced PC hydrolysis took place before significant inhibition of DNA synthesis occurred.

Discussion

We have demonstrated that CDDP treatment caused an increase in PC-PLC activity to yield an increase in PC turnover and DAG production and that CDDP treatment caused a decrease in IP_3 production but had no effect on PKC activity in a human non–small cell lung cancer cell line.

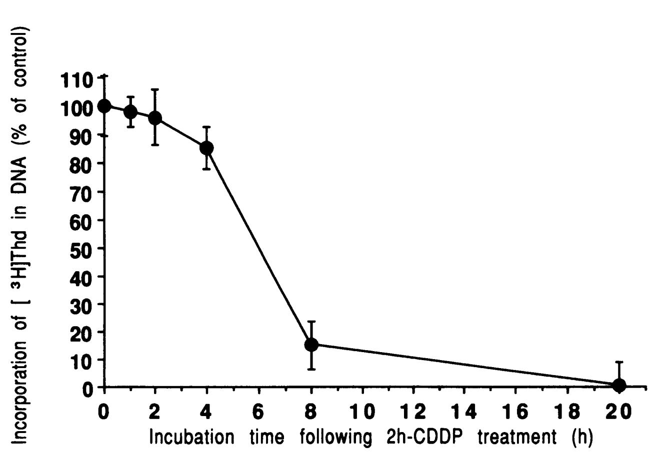

Figure 7. Inhibition of DNA synthesis in PC-9 cells at various times after 2 h treatment of CDDP. PC-9 cells (2 × 10^6 cells) were treated for 2 h with 20 μg/ml of CDDP (●) or PBS (○) as control. After 2 h, CDDP was removed and then cells were incubated in the complete medium for 0–18 h. Cell were labeled with 2 μCi of [^{3}H]Thd for 30 min at indicated time points. DNA synthesis was measured by determination of [^{3}H]Thd incorporation.

DAG is considered to be an important intermediate in signal transduction pathways, regulating cell growth and transformation (41), but most studies focused on its role in positive regulation on cell proliferation. Issandou et al. (42) have reported that permeant diacylglycerol 1,2-diotanoyl-*sn*-glycerol (DiC8) had a growth inhibitory effect on an MCF-7 breast cancer cell line and that DiC8 mimicked the effects of TPA on cell growth inhibition. We have previously demonstrated that CDDP-resistant PC-9 cells, PC-9/CDDP, showed cross-resistance to the growth inhibitory effect of TPA. These results suggested that DAG had a potential role in the negative regulation of cell proliferation as TPA had in some cells and that DAG had some role in CDDP-induced cytotoxicity.

We have demonstrated increased DAG production and decreased IP_3 production in PC-9 cells after CDDP treatment. We could not show an increase in PKC activity although an increase in DAG has occurred. If the source of DAG was only PI, these results would be contradictory considering the known characteristics of PI-derived DAG. However, recent evidence has demonstrated the existence of another phospholipid pathway leading to DAG production (20, 43, 44). PC-PLC–mediated hydrolysis of PC is now thought to be another important source of DAG (45, 46). And PC-derived DAG has been shown to have different fatty acid composition (47) and functions (48, 49). Although the distinct role of PC-derived DAG is not known, recent studies have demonstrated that PC-derived DAG did not cause PKC activation in vivo (50, 51). Further support for these results is that we have demonstrated increased PC-PLC activity to yield increased DAG production and no change of PKC activity after CDDP treatment. Our results suggest a novel function of PC-derived DAG.

Although we did not examine PI-PLC activity directly, it might be inhibited by CDDP treatment in that a decreased IP_3 production was observed in the present study. Recent reports have shown that analogues of PI such as hexachlorocyclohexanes (52) and manoalide (53) inhibited PI-PLC activity and caused a growth inhibition of tumor cells (54–56). These results suggested inhibition of PI-PLC activity and subsequent inhibition of PI turnover were important processes in the negative regulation of cell growth.

The mechanism whereby CDDP increases PC-PLC activity in PC-9 cells remains to be clarified. The activation mechanism of CDDP on PC-PLC could be through a direct effect of CDDP on the enzyme or substrate or through an influence on the regulatory mechanisms for PC-PLC. Some GTP-binding protein (G protein) has been suggested to be involved in the coupling of various agonist receptors to PI-PLC (57) and pertussis toxin; i.e., it interferes with the receptor-linked PI-PLC reaction in some tissues (12). On the other hand, there is some evidence to support the involvement of a G protein in receptor-dependent activation of PC breakdown by PLC (58) and phospholipase D (39, 45, 46, 59, 60). We have preliminary checked the effect of CDDP on GTPγS binding and ribosylation of G proteins by pertussis toxin. CDDP modulated neither GTPγS binding nor ribosylation of G proteins. And α and β subunits of G proteins were analyzed by immunoblotting in PC-9 and PC-9/CDDP cells, in which CDDP showed no effect on PC-PLC (data not shown). There was no difference in expression of α and β subunits of G proteins between PC-9 and PC-9/CDDP cells. According to these results, we have been considering that there is less possibility for CDDP to act on G proteins. However, recent reports demonstrated that PLC-mediated PC hydrolysis was through a G protein insensitive to pertussis toxin (61). And in some systems a pertussis toxin-sensitive GTP-binding protein is not involved in the coupling (62). Further investigation is necessary for the relationship between G proteins and PC-PLC.

Considering that CDDP-induced PC hydrolysis took place before a significant inhibition of DNA synthesis and that *trans*-DDP could not induce PC-PLC activation, PC-PLC activation and subsequent hydrolysis of PC might be important steps for CDDP-induced cytotoxicity. Further support for this comes from our preliminary data that 20 μg/ml of CDDP, which caused a significant increase in PC-PLC activity and in DAG production in the PC-9 cells, did not cause the same effect in $\sim$ 30-fold CDDP-resistant PC-9/CDDP cells. This suggests that change in phospholipid metabolism, described in this report, might contribute to the mechanism of acquired CDDP resistance and reinforces our hypothesis that the change of phospholipid metabolism might related to CDDP-induced cytotoxicity.

Almost all of the previous studies investigating the relationship between anticancer agent sensitivity (or cytotoxicity) and signal transduction pathways have focused only on the change of PKC and/or PI turnover. However, the results presented here suggest that PC metabolism might have an important role in anticancer drug–induced cytotoxicity.

The CDDP effect on PC metabolism seems to mimic to the effect of IL-1 (63), IL-3 (64), and Interferon α (65). In interferon α, PC-hydrolysis is coupled to the growth inhibitory effect. Considering these evidences, we could speculate about the possibility for the combination of CDDP and these compounds. Recently, it has been demonstrated that many lipid compounds, including phosphatidylcholine analogues and ether-lipids analogues, have antitumor effects against several kinds of tumor cells (66). These compounds were also expected to be used in the combination with CDDP.

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