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Alteration of Type II Regulatory Subunit of cAMP-dependent Protein Kinase in Human Cisplatin-resistant Cells as a Basis of Collateral Sensitivity to 8-Chloro-cAMP

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A cyclic adenosine 3′,5′-monophosphate (cAMP) analogue, 8-chloro-cAMP (8-Cl-cAMP), had a collateral growth-inhibitory effect on a *cis*-diamminedichloroplatinum(II) (CDDP)-resistant human cancer cell lines (PC-14/CDDP). The non-selective analogues dibutyryl-cAMP, 8-bromo-cAMP and forskolin, which are cAMP agonists, showed far less cytotoxicity than 8-Cl-cAMP in both cell lines. There was no significant difference in cAMP content between PC-14 and PC-14/CDDP. Because 8-Cl-cAMP has been shown to bind selectively to the site I receptor of the type II regulatory subunit (RII) of cAMP-dependent protein kinase, we determined the level of expression of regulatory subunits in PC-14 and PC-14/CDDP cells by photoaffinity labeling. PC-14/CDDP cells had a higher RII level, low site I receptor of type I regulatory subunit (RI) level, and a lower RI/RII ratio than the parental PC-14 cells. Exposure to 8-Cl-cAMP increased the RI and RII level in PC-14/CDDP cells in dose- and time-dependent manners. On the other hand, in parental PC-14 cells, RII was not detected and the levels of RI and RII were not increased by exposure to 8-Cl-cAMP. These results suggested that the change in RI and/or RII levels caused by 8-Cl-cAMP was correlated with 8-Cl-cAMP-induced growth inhibition and that the collateral sensitivity to 8-Cl-cAMP in CDDP-resistant cells was due to the increased RII level. Our results suggest that 8-Cl-cAMP can be used in combination with CDDP and that measurement of RI and RII levels and/or the RI/RII ratio is a useful tool to predict CDDP sensitivity.

Key words: Cisplatin — 8-Cl-cAMP — Cyclic AMP-dependent protein kinase

cis-Diamminedichloroplatinum(II) (CDDP)[6] is one of the most potent anticancer agents. To elucidate the mechanism of CDDP-resistance, we have established CDDP-resistant sublines *in vitro* from non-small cell lung cancer cell lines.[1)] Mechanisms of CDDP-resistance are considered to be multifactorial.[2)] The main mechanism has not yet been determined; however, the CDDP-resistance appears to be quite different from the multidrug resistance phenotype. Some studies suggest that a signal transduction pathway mediated by protein kinase C might be involved in CDDP cytotoxicity or CDDP-resistance, although the mechanism is still unclear.[3-6)] We recently reported increased phosphorylation of some phosphoproteins in a CDDP-resistant human lung cancer cell line.[7)] However, we did not observe any difference in protein kinase C activity between the parent and its CDDP-resistant cell line. We speculated that a protein kinase other than kinase C might be involved in CDDP-resistance. A relationship between the adenosine 3′,5′-monophosphate (cAMP)-dependent signaling pathway and CDDP-induced growth inhibition has not been reported. We determined the cytotoxicity of several drugs which could act on cAMP or cAMP-dependent kinase in CDDP-resistant cells in order to elucidate the mechanism of CDDP-resistance.

We found that 8-chloro-cAMP (8-Cl-cAMP), which selectively binds to the site I receptor of the type II regulatory subunit (RII), had a greater inhibitory effect on CDDP-resistant cells than on their parental cells. The mechanisms of the collateral sensitivity to 8-Cl-cAMP in the CDDP-resistant cell line were analyzed.

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[6] The abbreviations used are: CDDP, *cis*-diamminedichloroplatinum(II); cAMP, cyclic adenosine 3′,5′-monophosphate; 8-Cl-cAMP, 8-chloro-cAMP; RI, site I receptor of type I regulatory subunit; RII, site I receptor of type II regulatory subunit; PBS, phosphate-buffered saline; FBS, fetal bovine serum; 8-azido-N3-[^{32}P]cAMP: 8-azidoadenosine-3′,5′-cyclic [^{32}P]monophosphate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PAG; polyacrylamide gel; 8-Br-cAMP, 8-bromo-cAMP; Mr, molecular weight; PSL, photostimulated luminescence.

MATERIALS AND METHODS

Chemicals CDDP was obtained from Bristol-Myers Squibb Japan (Tokyo). 8-Cl-cAMP and RPMI 1640, calcium-free and magnesium-free Dulbecco's phosphate-buffered saline (PBS), and fetal bovine serum (FBS) was provided by the Research and Development Laboratory, Tonen Co. (Tokyo), Nissui Pharmaceutical Co. (Tokyo), Daiichi Pure Chemicals Co. (Tokyo) and Bio-beck Laboratories, Co. (Toronto, Canada), respectively. 8-Azidoadenosine-3′,5′-cyclic [^{32}P]monophosphate (8-azido-N3-[^{32}P]cAMP) was purchased from ICN Radio-chemicals (Irvine, CA). Other drugs and chemicals were purchased fron Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

Cell cultures PC-14 and PC-9 are human non-small cell lung cancer cell lines. H69 is a human small cell lung cancer cell line. The CDDP-resistant sublines, PC-14/CDDP, PC-9/CDDP, and H69/CDDP were established by exposure of these parental cell lines to stepwise increasing CDDP concentrations and were selected by the limiting dilution technique.[1] Cells were grown in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified 5% CO_2 atmosphere at 37°C.

Growth-inhibition assay To determine the growth-inhibitory effect of the drugs, we used the tetrazolium dye assay of Mosmann.[8] Briefly, 200-μl aliquots of a suspension of exponentially growing cells (5×10^4/ml) were seeded in 96-well microtiter plates and incubated for 12 h. Ten-μl aliquots of the drugs at various concentrations were added. After exposure to the drugs for 72–96 h, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetraazolium bromide (MTT) solution (5 mg/ml in distilled water) was added to each well and the plates were incubated at 37°C for another 4 h. After centrifugation of the plates at 800g for 5 min, the medium was aspirated from each well as completely as possible. Two hundred μl of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan. The optical density was measured at 562 and 630 nm using a Δ soft ELISA analysis for a Macintosh computer interfaced to a Bio-Tek Microplate Reader (EL-340, Bio Metallics, Princeton, NJ). As a control, a well containing only RPMI-FBS and MTT was used. Each experiment was performed in triplicate and carried out 3 times or more independently. The IC_{50} was defined as the concentration required for 50% reduction of the optical density in each test and was calculated as (mean absorbance in 3 wells containing drug − absorbance in 3 control wells)/(mean absorbance in 3 drug-free wells − absorbance in 3 control wells)$\times$100. Relative resistance was defined as the IC_{50} for the resistant subline/IC_{50} for the parental cell line.

cAMP assay Cells were grown to confluence in tissue culture dishes and harvested by 5-min trypsinization at 37°C. Samples were harvested at indicated times by rapid centrifugation and addition of 150 μl of 50 mM Tris-4 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N-tetraacetic acid to the pellet. The cells were homogenized by sonication at the lowest setting for 20 s and deproteinized by incubation for 3 min in a boiling water bath. Supernatants were assayed for cAMP with a kit from Amersham, based on a competitive protein binding assay.[9] cAMP levels were expressed as pmol/mg protein.

Photoaffinity labeling of cAMP receptor proteins Growing cells (5×10^6/ml) were exposed to various concentrations of 8-Cl-cAMP in 60-mm dishes, and 24 h later the cell suspensions were centrifuged and washed with ice-cold PBS(−). For the time course study, cells were exposed to 20 μM 8-Cl-cAMP for the indicated duration (6, 12, 24, 48 h). The cell pellets were suspended in 100 μl of buffer TEN (0.1 M NaCl, 5 mM $MgCl_2$, 1% Nonidet P-40 (Iwai Chem. Co., Tokyo), 0.5% deoxycholate, 20 mM Tris-HCl, pH 7.4) for 30 min at 4°C and centrifuged at 3000g for 5 min according to the method of Katsaro *et al.*[10] The supernatant was used for photoaffinity labeling in the following, experiment. Photoactivated incorporation was performed as described by others.[3, 4, 11] Briefly, photoactivated incorporation of 8-N3-[^{32}P]cAMP was conducted in 30 μl of buffer TEN. For the competitive inhibition assay, several concentrations of cAMP were added to the mixtures. Each mixture was held at 4°C for 30 min in the dark and irradiated with UV light (254 nm) for 5 min. After addition of 10 μl of 4$\times$ sodium dodecyl sulfate (SDS)-sample buffer, the mixture was boiled and subjected to electrophoresis in a 10% SDS-polyacrylamide gel (PAG PLATE 10, Daiichi Pure Chemicals). The separated proteins were transferred to a PVDF membrane (Millipore Corp., Bedford, MA), and exposed to an imaging plate (BAS-III, Fuji Photo Film Co. Ltd., Tokyo). The plate was analyzed by a Bio-image analyzer BAS 2000 (Fuji Photo Film Co. Ltd.).

RESULTS

Inhibitory effect of 8-Cl-cAMP on cell growth There was no significant difference of growth rate between the parental (PC-14, PC-9, and H69) and their respective CDDP-resistant (PC-14/CDDP, PC-9/CDDP, HL69/CDDP) cell lines (data not shown). The inhibitory effects of CDDP and 8-Cl-cAMP are summarized in Table I. The three different CDDP-resistant cell lines showed higher sensitivity to 8-Cl-cAMP compared with their parental cell lines. On the other hand, forskolin, 8-butyryl-cAMP, and 8-bromo cyclic adenosine monophosphate (8-Br-cAMP) caused far less growth inhibi-

Table I. Growth-inhibitory Effects of CDDP and 8-Cl-cAMP on Human Lung Cancer Cell Lines and Their CDDP-resistant Cells

Cell line	IC_{50} (μM)[a]	
	CDDP (RR)[b]	8-Cl-cAMP (RR)
PC-14	0.29[c]	5.82
PC-14/CDDP	2.23 (×7.8)	0.94 (×0.16)
PC-9	0.32	4.87
PC-9/CDDP	7.14 (×22.3)	1.13 (×0.23)
H69	0.26	9.22
H69/CDDP	2.65 (×10.2)	1.82 (×0.20)

a) Drug concentration that inhibits cell growth by 50%.
b) Relative resistance (RR) value equals the IC_{50} of the resistant cell line divided by the IC_{50} of the parental cell line.
c) Each value is the mean of 3 independent experiments.

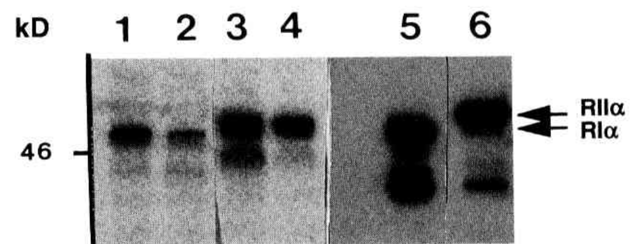

Fig. 1. Photoaffinity labeling of RI and RII in PC-14 and PC-14/CDDP cells. Lane 1, PC-14 cell; lane 2, PC-14 cells with 100 p*M* cAMP; lane 3, PC-14/CDDP cell; lane 4, PC-14/CDDP cells with 100 p*M* cAMP; lane 5, RI*α*, rabbit RI (Sigma); lane 6, RII*α*, bovine RII (Sigma). Cell lysate was prepared and labeled with 8-N3-[^{32}P]cAMP as described in "Materials and Method."

Table II. Basal cAMP Content of Parental and CDDP-resistant Cells

Cell line	pmol/mg protein[a]
PC-14	10.4±0.33 (8)
PC-14/CDDP	10.9±0.42 (8)
PC-9	10.5±0.31 (6)
PC-9/CDDP	10.1±0.60 (6)
H69	8.5±0.67 (6)
H69/CDDP	8.7±0.70 (6)

a) Data are the mean±SE of the indicated number of determinations (N).

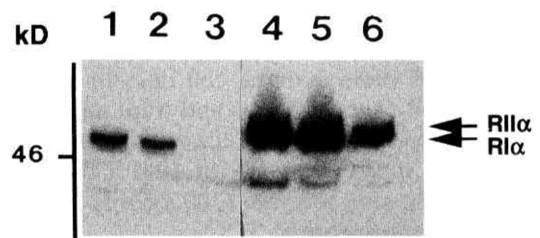

Fig. 2. Effect of cold cAMP on the formation of the RI and RII cAMP regulatory subunits of PC-14 (lanes 1–3) and PC-14/CDDP (lanes 4–6). A cell lysate was prepared and labeled with 8-N3-[^{32}P]cAMP in several concentrations of cold cAMP as described in "Materials and Methods." Lane 1, a cell lysate from PC-14 cells in the absence of cAMP; lane 2, a cell lysate from PC-14 cells in the presence of 20 p*M* cAMP; lane 3, a cell lysate from PC-14 cells in the presence of 200 p*M* cAMP; lane 4, a cell lysate from PC-14/CDDP cells in the absence of cAMP; lane 5, PC-14/CDDP cells, a cell lysate from PC-14 cells in the presence of 20 p*M* cAMP; lane 6, PC-14/CDDP cells in the presence of 200 p*M* cAMP.

tion of these cell lines (data not shown). Non-cytotoxic doses of 8-Cl-cAMP did not modulate the cytotoxicity of CDDP (data not shown).

cAMP content We thought that the cAMP level might influence the growth inhibitory effect of 8-Cl-cAMP, and therefore, we compared the cAMP content of the parental and CDDP-resistant cell lines (Table II). There was no significant difference in cAMP content between them. This result suggests that cellular cAMP content was not correlated with the growth-inhibitory effect of 8-Cl-cAMP.

Expression of RI and RII cAMP regulatory subunits in CDDP-resistant cells 8-Cl-cAMP has been demonstrated to bind RII (and weakly to RI) and to activate cAMP-dependent protein kinases.[12] We speculated that the collateral sensitivity of 8-Cl-cAMP in CDDP-resistance was due to change in the regulatory subunits. We examined the PC-14/CDDP cell line for the changes in RI and RII of cAMP-dependent protein kinase because this cell line showed the highest collateral sensitivity level among the cell lines tested. The levels of RI and RII cAMP regulatory subunits were measured by using the photoaffinity ligand 8-azido-N3-[^{32}P]cAMP.

Parental PC-14 cells and CDDP-resistant PC-14/CDDP cells contain cAMP receptor proteins. As shown in Fig. 1, PC-14 cells (lane 1) contain a major cAMP receptor protein with a molecular weight (Mr) of 48,000. The 48,000-Mr protein appears to be RI*α*, because it comigrated in SDS-PAGE with the purified preparation of the 48,000-Mr RI*α* from rabbit skeletal muscle (line 5).[13] In the presence of 100 p*M* cAMP, the incorporation of radioactivity was decreased but not abolished (lane 2). PC-14/CDDP cells contained a major cAMP receptor protein with an Mr of 56,000. This protein

Table III. Relative Levels of cAMP Receptor Proteins in PC-14 and PC-14/CDDP

Cell line	RI	RII	RI+RII	RI/RII
PC-14	11980±1055[a]	1771±136[a]	13751±1552[b]	6.7[a]
PC-14/CDDP	1841±233[a]	9250±1008[a]	11091±1085[b]	0.19[a]

a) No significant difference between PC-14 and PC-14/CDDP cells by unpaired Student's *t* test.
b) Significant difference between PC-14 and PC-14/CDDP cells by unpaired Student's *t* test ($P<0.01$).
The radiation doses of areas corresponding to RI and RII subunits were counted by using the BAS 2000. The levels of the receptor proteins are expressed as PSL value minus background. The scale was expanded to differentiate the lower intensity bands. The data are average value±SE of six experiments.

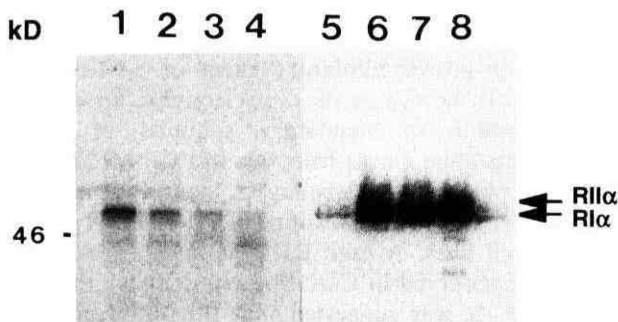

Fig. 3. Effect of 8-Cl-cAMP on the levels of the RI and RII cAMP regulatory subunits of PC-14 (lanes 1–4) and PC-14/CDDP (lanes 5–8). Cells were exposed to various concentrations of 8-Cl-cAMP for 24 h. A cell lysate was prepared and labeled with 8-N3-[^{32}P]cAMP as described in "Materials and Methods." Lane 1, PC-14 cells; lane 2, PC-14 cells treated with 12.5 n*M* 8-Cl-cAMP; lane 3, PC-14 cells treated with 50 n*M* 8-Cl-cAMP; lane 4, PC-14 cells treated with 200 n*M* 8-Cl-cAMP; lane 5, PC-14/CDDP cells; lane 6, PC-14/CDDP cells treated with 12.5 n*M* 8-Cl-cAM; lane 7, PC-14/CDDP cells treated with 50 n*M* 8-Cl-cAM; lane 8, PC-14/CDDP cells treated with 200 n*M* 8-Cl-cAM.

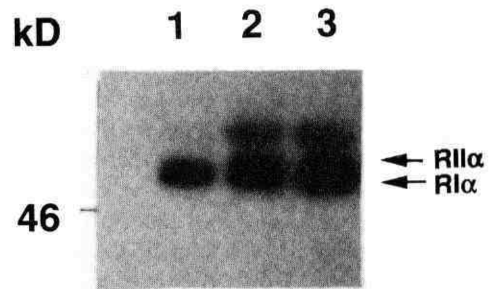

Fig. 4. Time-dependent formation of RI and RII by 8-Cl-cAMP in PC-14/CDDP cells. Cells were exposed to 200 n*M* 8-Cl-cAMP for 6 (lane 1), 12 (lane 2), or 24 h (lane 3). A cell lysate was prepared and labeled with 8-N3-[^{32}P]cAMP as described in "Materials and Methods."

appears to be the RII*α* of the cAMP receptor protein, because it comigrated in SDS-PAGE with the 56,000-Mr RII*α* from bovine heart (line 6).[13, 14] In lane 3, lower Mr bands were also observed and the band was decreased by addition of 100 p*M* cAMP (lane 4). Thus, the lower band is considered to be a degradation product. To confirm that the 48,000 and 56,000 bands are RI and RII, we examined the competitive inhibition assay using cold cAMP (Fig. 2). The incorporation of radioactivity was unaffected by 20 p*M* cAMP (Fig. 2, lanes 2 and 5 vs. 1 and 4). The incorporations of radioactivity in the 48,000 and approximately 56,000 bands were decreased by the presence of 200 p*M* cAMP (Fig. 2, lanes 3 and 6). The incorporations of radioactivity in lower bands were also decreased by 200 p*M* cAMP in both PC-14 and PC-14/CDDP cells. The results suggest that the higher prominent bands of 48,000 and 56,000 Mr have a cAMP binding site while the lower bands are probably degradation products.

We measured the radioactivity of the RI and RII subunits using a BAS 2000 image analyzer. The radiation doses were expressed as the photostimulated luminescence (PSL) value minus background. As shown in Table III, PC-14 cells have a high RI level and the RI/RII ratio is 5.02. PC-14/CDDP cells have a high RII level and the RI/RII ratio is 0.68. There were marked differences in the levels of RI and RII between PC-14 and PC-14/CDDP cells.

Effect of 8-Cl-cAMP on the levels of RI and RII cAMP regulatory subunits We compared the effect of 8-Cl-cAMP on the levels of the regulatory subunit of PC-14 and PC-14/CDDP (Fig. 3). In the untreated cells of both lines, the major 48,000-Mr protein of RI was observed (lanes 1 and 5). In PC-14 cells, 8-Cl-cAMP decreased the RI band as compared with the untreated cells in a dose-dependent manner (lanes 1–4). In the PC-14/CDDP cells, two major bands of Mr 48,000 and 56,000, which appear to be RI*α* and RII*α*, respectively, were markedly increased by exposure to 8-Cl-cAMP as compared with the untreated cells (lanes 5–8). This

increase in both subunits was not observed in PC-14 cells. 8-Cl-cAMP increased the levels of both subunits only in PC-14/CDDP. These results suggested that the differences in sensitivity of PC-14 and PC-14/CDDP cells to 8-Cl-cAMP are due to change in the levels of RI and RII or the RI/RII ratio.

We also examined the time-dependent formation of RIα and RIIα in PC-14/CDDP cells (Fig. 4). 8-Cl-cAMP increased RI and RII formations in a time-dependent manner in PC-14/CDDP cells (Fig. 4, lanes 1–4).

DISCUSSION

The primary mediator of cAMP action in eukaryotic cells is cAMP-dependent protein kinase,[15, 16] which is composed of two catalytic and two regulatory subunits, RI and RII. cAMP binds to the two different regulatory subunits (RI and RII), induces a comformational change, and activates the catalytic subunit. On the basis of these distinct regulatory subunits, cAMP-dependent protein kinase is divided into two isozymes, type I and type II. A differential effect of cAMP on cell growth, differentiation and carcinogenesis, for example, may be brought about through the different regulatory subunits.[17, 18] It has been reported that 8-Cl-cAMP, which selectively binds to the site I receptor of RII,[19] suppresses the growth of breast cancer and colon cancer cells and a leukemia cell line,[20] as well as the growth of human cancer xenografts of various cell types, including breast, colon, and lung carcinomas, in athymic mice.[10, 12, 21–23] However, there are no reports about the relationship between these changes in cAMP receptors and anticaner-drug resistance.

In the present study, we demonstrated that CDDP-resistant cells showed collateral sensitivity to 8-Cl-cAMP, which is a site-selective cAMP analog, and we compared these regulatory subunits of cAMP-dependent kinases in CDDP-resistant cells with those of the parental cells. We speculated that the collateral sensitivity of CDDP-resistant cells to 8-Cl-cAMP might be due to the changes in type I and type II isozymes. This selective modulation of the RI and RII cAMP receptor proteins was not mimicked by the cAMP analogues previously studied (cAMP itself or agents that increase the cellular cAMP level). At high levels, cAMP, having no site selectivity,[24] activates both type I and type II isozymes maximally and equally without discrimination.[13, 25] In our cell line, in fact, the non-selective analogues dibutyryl-cAMP, 8-Br-cAMP and forskolin, which are cAMP agonists, showed far less cytotoxicity than 8-Cl-cAMP (data not shown). These results are consistent with the results of previous studies showing that 8-Br-cAMP is less inhibitory to growth than 8-Cl-cAMP.[23] Therefore, our results support the concept of a relationship between the site selectivity and the growth-inhibitory effect of 8-Cl-cAMP.

On the other hand, changes in cAMP-dependent protein kinase activity have been reported for human thyroid carcinomas,[26] human cerebral tumors,[27] and leukemia cells from patients.[28] We had thought that the cAMP level might influence the growth-inhibitory effect of 8-Cl-cAMP in PC-14 and PC-14/CDDP cell lines. We checked the cAMP content in PC-14 and PC-14/CDDP cell lines. However, there was no significant difference between them (Table II). This result suggests that the cellular cAMP content does not influence the growth-inhibitory effect of 8-Cl-cAMP.

Since the growth-inhibitory effect of 8-Cl-cAMP is considered to be due to its site selectivity, we analyzed the expression of regulatory subunits of cAMP-dependent protein kinase receptors in PC-14/CDDP and PC-14 by photoaffinity labeling. A significant difference was observed in the expressions of RI and RII between the two cell lines. A high RII level and a low RI/RII ratio were observed in CDDP-resistant cells (Fig. 1 and Table III). It was suggested that this difference could explain the difference in sensitivity to 8-Cl-cAMP between PC-14 and PC-14/CDDP. cAMP-dependent protein kinase is usually present in tissues as a mixture of type I and type II isozymes.[29–31] The cAMP-dependent protein kinase type I and type II ratio varies among tissues and the increased expression of protein kinase type I or type II is correlated with active cell growth, cell transformation, or differentiation, suggesting that one or both of the cAMP-dependent protein kinase isozymes may be involved in malignant cell growth.[18] The relative content of protein kinase type I and type II varies among tissues in adult animals and also in the same tissue among different species of animals,[29–31] although the total R subunit/C subunit molar ratio in all normal tissues examined was found to be 1:1.[31]

We demonstrated that 8-Cl-cAMP increased both the RI and RII levels in PC-14/CDDP and decreased the RI level in PC-14 cells (Fig. 3). We considered that the high level of RII and/or the low RI/RII in PC-14/CDDP cells was responsible for their high sensitivity to 8-Cl-cAMP. On the other hand, 8-Cl-cAMP did not enhance the level of RII but decreased the RI level in parental PC-14 cells. This finding suggested that the RI level was not responsible for the growth-inhibitory effect of 8-Cl-cAMP. Selective modulation of type II versus type I protein kinase isozymes was correlated with the degree of growth inhibition.

It was concluded that the cellular machanism of collateral sensitivity to 8-Cl-cAMP in CDDP-resistant cells was due to the change in the RI and/or RII level and the low RI/RII rartio and that the agonistic effect of 8-

Cl-cAMP on RII but not on RI was responsible for the growth inhibition. Collateral sensitivity to 8-Cl-cAMP was observed in all 3 CDDP-resistant cell lines tested. It seemed possible that this observation could apply to all CDDP-resistant cells. We compared the growth-inhibitory effect of 8-Cl-cAMP on two other drug-resistant, i.e., doxorubicin-(K562/ADM)[32] and etoposide-resistant (H69/VP),[33] cell lines with the effect on the parental K562 and H69 cells, respectively. There was no difference in the growth-inhibitory effect on the resistant and parental cell lines (data not shown). These results support the idea that the collateral sensitivity is specific to CDDP-resistance.

Recently Mann *et al.* demonstrated that forskolin modulated CDDP accumulation and sensitivity in parental and CDDP-resistant ovarian carcinoma cell lines.[34] They suggested that cAMP influenced the CDDP accumulation. In our experiment, we did not observe a modulatory effect of forskolin on CDDP cytotoxicity, or find a significant difference in cAMP content between parental and CDDP-resistant cells. Therefore, the collateral sensitivity to 8-Cl-cAMP in this study is considered to be due to a different mechanism from the modulation by cAMP or a cAMP agonist such as forskolin.

In view of the present results, we intend to investigate (1) the synergistic effect of CDDP and 8-Cl-cAMP, (2) combination therapy with CDDP and 8-Cl-cAMP, (3) secondary choice of 8-Cl-cAMP after CDDP treatment, (4) prediction of CDDP sensitivity by cAMP-receptor analysis, and (5) the design of new drugs targeted at site-selective analogues.

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