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Signal transduction plays a crucial role in carcinogenesis. A defect in signaling, by evading cell death or promoting cell proliferation, may result in neoplastic transformation or protection of cells from the cytotoxicity of anticancer drugs. Therefore, in order to understand the complex mechanism of drug resistance, it is relevant to probe into the important signal transduction pathways. Protein kinase C, a key signal transducer, influences cisplatin sensitivity in many cell lines. We examined whether or not the PKC signal transduction pathway is affected during development of resistance to cisplatin by tumor cells. PKC activators increased cisplatin sensitivity in both parental and cisplatin-resistant cells. Western blot analysis showed a slight decrease in cPKCα and nPKCε, an elevation in nPKCδ and no change in the abundance of aPKCζ in HeLa/CP cells compared to HeLa cells. Though TPA-induced translocation of PKC isoforms was identical in both cell lines, down regulation of PKCδ was defective in resistant cells. Therefore, a deregulation in PKCδ was associated with cisplatin resistance.

CHARACTERIZATION OF PROTEIN KINASE C IN CISPLATIN-SENSITIVE AND –RESISTANT HUMAN CERVICAL CANCER HeLa CELLS

Sanghamitra Mohanty, MBBS

APPROVED:

Major Professor

Committee Member

Committee Member

Chair, Department of Molecular Biology and Immunology

Dean, Graduate School of Biomedical Sciences

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Sanghamitra Mohanty, MBBS.

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CHAPTER I

INTRODUCTION

The coordinated transduction of extracellular signals into intracellular events results in normal cell growth, differentiation, and cell death. Disturbances in the cellular signaling pathways may lead to uncontrolled cell proliferation or inadequate cell death, resulting in malignancies. Anticancer drugs primarily kill cancer cells by inhibiting cell proliferation. However, one of the problems with conventional cancer therapy is the development of drug resistance.

It is now recognized that various signal transduction pathways determine the vulnerability of neoplastic cells to cytotoxic drugs. Disturbances in signal transduction pathways that contribute to neoplastic transformation by antagonizing apoptosis may at the same time protect cells from the cytotoxic effects of the anticancer drugs (24). Thus, intervention at the level of signal transduction could potentially reverse drug resistance (24).

Protein kinase C (PKC), a family of phospholipid-dependent serine/threonine kinases, plays a key role in cell signaling. The first link between PKC and signal transduction was demonstrated in the 1980s by the fact that diacylglycerol (DAG) is the physiological stimulator of PKC (7). Since then, the importance of PKC has been unequivocally shown to modulate a wide range of cellular functions. When

hormones, neurotransmitters, mitogens or growth factors interact with their cell surface receptors, phospholipase C gets activated and generates inositol trisphosphate (IP_3) and DAG from phosphatidylinositol 4,5-bisphosphate (PIP_2). IP_3 then induces the release of calcium from the endoplasmic reticulum. DAG and Ca^{2+}, together with membrane phospholipids, activate PKC. Once activated, PKCs phosphorylate their substrates on serine and threonine residues and trigger many cellular responses including cell proliferation, differentiation, membrane transport and gene expression (9).

PKC is a multigene family of 12 isoforms which can be categorized into 3 subgroups based on their structural similarities and cofactor requirements: Group A or conventional PKC (cPKC): $\alpha, \beta I, \beta II, \gamma$; Group B or novel PKC (nPKC):$\delta, \varepsilon, \theta, \eta$ (L) and Group C or atypical PKC (aPKC): λ, ζ, ι. In addition, PKCμ structurally resembles nPKCs, but functionally behaves as aPKCs (7). These isoforms differ in their biochemical properties, tissue specific expression, intracellular localization, substrate specificity and function (10). cPKCs are DAG sensitive and Ca^{2+} dependent and nPKCs are DAG sensitive but Ca^{2+} independent. aPKCs are both DAG and Ca^{2+} insensitive and only activated by the membrane phospholipid, phosphatidylserine.

PKC is the intracellular receptor for tumor promoting phorbol esters (11) which stimulate it by a mechanism similar to DAG. While stimulation of PKC by DAG is transient, phorbol esters cause persistent activation. Extended exposure to these compounds leads to proteolytic degradation or down regulation of PKC (13).

Other PKC activators of importance are bryostatin I and indolactam V (ILV). ILV is an indole alkaloid which competes for phorbol ester binding site on PKC (7,25). Bryostatin 1 binds to and activates PKC in a manner similar to phorbol esters, but antagonizes some of the effects of the latter. Therefore, bryostatin 1 is considered as a partial agonist of PKC. Another major difference between bryostatin 1 and phorbol esters is that, bryostatin has been demonstrated to be anti-neoplastic in murine melanoma, murine leukemia, and ovarian sarcoma (14).

Signal transduction via PKC is closely regulated by its subcellular localization (12). PKC is usually cytosolic, but upon activation, the enzyme is redistributed from the cytosol to the membrane fractions. This translocation to different subcellular locations is specific for each PKC isoform. Translocated PKCs then phosphorylate their substrates, thereby triggering the appropriate cellular response.

Several lines of evidence indicate that, modulation of PKC activity can alter cellular sensitivity to cytotoxic agents (26). A close relationship between PKC activity and cellular sensitivity to one of the prominent anticancer drug, cisplatin, has been shown previously (6,10,13,14). Cisplatin [*cis*-Diamminedichloro-platinum (II)] is a neutral square planar coordination compound. The two chloride ligands are stable at extracellular chloride concentrations (~100 mM), but after diffusion into a cell, the lower chloride concentration (~3 mM) facilitates exchange of the chloride ions for water or hydroxyl groups (3). This exchange produces a bifunctional charged electrophile that can react with any nucleophile including the sulfhydryl groups on proteins and nucleophilic groups on nucleic acids (4).

Cisplatin is widely used in the treatment of many solid tumors including testicular, ovarian, bladder, esophageal, and cervical cancer. The cytotoxicity of cisplatin is believed to be due to the formation of DNA adducts, which include DNA-protein cross-links, DNA monoadducts, and interstrand and intrastrand DNA cross-links. Quantitative studies show that 1,2-intrastrand d(GpG) cross-links account for 65% of all platinum-DNA adducts formed in vitro (4). By forming DNA adducts, cisplatin inhibits DNA replication and chain elongation. These effects on DNA synthesis are believed to be the main cause of the cytotoxic effects of cisplatin (5). However, recent experiments have indicated that the ability of cisplatin to inhibit DNA synthesis is not always the only mechanism of cytotoxicity (3). Indeed, the analysis of cell death induced by cisplatin reveals DNA fragmentation into multimers of 180 base pairs, followed by loss of membrane integrity and cell shrinkage which are consistent with programmed cell death or apoptosis (4). Additionally, only a small fraction (~1%) of cisplatin actually interacts with DNA, therefore other cellular factors can contribute to its cytotoxicity (4).

The preeminent role of cisplatin has led to the development of new management strategies that focus on cisplatin based drug combinations and earlier intervention with this compound (1). Unfortunately however, the effectiveness of chemotherapy is compromised by the development of drug resistance. Even a small increase in resistance of a tumor cell to cisplatin is clinically important, as dose escalation leads to severe neuro- and nephrotoxicity.

Several potential mechanisms of resistance, including decreased drug accumulation, increased drug detoxification, enhanced DNA repair, increased levels of intracellular thiols and increased ability of cells to tolerate damaged DNA have been shown to contribute to cisplatin resistance (6). However, these mechanisms do not explain the observed degree of resistance in many cases. For instance, in A2780-C30 cell lines, the decrease in drug uptake was shown to be minimal, whereas cisplatin resistance was 34-280-fold (4). Additionally, increased glutathione levels have been found in some cisplatin-resistant cells, but not in others.

As it has already been discussed, cells treated with cytotoxic levels of cisplatin display the biochemical and morphological features of apoptosis (4). Therefore, the resistance to cisplatin may be caused by a deregulation of the cell death machinery.

Cellular sensitivity to cisplatin can be greatly influenced by the PKC signal transduction pathway (6). Activators of PKC such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA) has been shown to enhance cisplatin sensitivity in small cell lung cancer, ovarian cancer, human cervical cancer and rat Walker carcinoma cells (6). The enhancement in antiproliferative activity of cisplatin by various concentration of TPA paralleled the increase in PKC activity in HeLa cells (13). Additionally, the ability of different phorbol esters to activate PKC correlated with their ability to decrease the IC_{50} for cisplatin (13). Another PKC activator, lyngbyatoxin A, which is structurally distinct from phorbol esters, also sensitized HeLa cells to cisplatin (14). However, the mechanism by which PKC influences

cisplatin sensitivity is unclear. In HeLa cells, phorbol ester-induced cisplatin sensitization appeared to be due to an increase in cellular platinum content (13). In the case of rat Walker carcinoma cells, depletion of PKC was associated with cisplatin sensitization, whereas in ovarian and cervical cancer, activation of PKC was related to cisplatin sensitization (6). Therefore, the role of PKC in cisplatin senstivity is controversial. However, it is clear that PKC positively or negatively regulates cisplatin sensitivity depending on the cell type. This complexity may also be explained to some extent by the fact that there are 12 PKC isoforms which differ in their biochemical properties, tissue specific expression, and function.

As the activation of PKC results in enhancement of cellular sensitivity to cisplatin, we investigated if an alteration in PKC signal transduction is associated with cisplatin resistance.

Significance of this research:

The effectiveness of cisplatin therapy is restricted by the emergence of resistant tumor cell populations. PKC activators significantly increase cisplatin sensitivity, thereby demonstrating the critical role of PKC in cisplatin-induced cytotoxicity. Hence, an understanding of the PKC signal transduction pathway in cisplatin-sensitive and –resistant cell lines may help in targeting the altered signals with a goal of reversing resistance to sensitivity.

CHAPTER 2

METHODS

Cell lines:

Human cervical carcinoma (HeLa) cells were used as the cisplatin-sensitive cell model. A cisplatin-resistant phenotype (HeLa/CP) was then developed from the HeLa cells by an *in vitro* selection process. Briefly, the HeLa cells were treated with escalating concentrations of cisplatin for several months and the drug-resistant colonies were selected.

Cell culture:

Cells were maintained in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 ug/ml streptomycin and kept in a humidified incubator at 37°C with 95% air and 5% CO_2.

Assessment of cell viability:

Exponentially growing cells (2000 cells/well) were plated in microtiter plates and incubated at 37°C in 5% CO_2. The following day, cells were pretreated without or

with PKC modulators for 24 h and then incubated with varying concentrations of cisplatin. Following a 24 or 48 h exposure to the anticancer drug, the number of viable cells was determined using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] as described by Carmichael *et al* (23). Control values were calculated for each individual experiment based upon the results obtained in the absence of cisplatin, but in the presence of solvent (Me_2SO) or the activator. For each experiment, results from at least 4 individual wells were used to determine the mean value.

Immunoblot analysis:

Following treatment with PKC modulators, cells were collected, washed twice with cold PBS, and lysed in 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1 mM EDTA, 1.0% Triton X-100, 0.5% Nonidet-40, 0.2 mM sodium vanadate, 5 mM benzamidine, and 20 ug/ml each of leupeptin, aprotinin, and soybean trypsin inhibitor. Equal amounts of protein from total cellular extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred electrophoretically to a polyvinylidene difluoride membrane. Immunoblot analyses were performed with 1:1000 dilution of various PKC isozyme-specific antibodies. The blots were visualized using the enhanced chemiluminescence detection reagents and the manufacturer's protocol. Intensities of immunoreactive proteins were quantified by densitometry. In each experiment, the same blot was stripped and probed with several antibodies.

Subcellular fractionation (27):

Cells were exposed to 100 nM TPA for 0-48 h. Then they were harvested, washed with cold PBS, and homogenized by syringing in 20 mM Tris-HCL (pH 7.5), 0.25 M sucrose, 2 mM EDTA, 5 mM EGTA, 10 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 10 ug/ml of leupeptin, aprotinin, and soybean trypsin inhibitor (buffer A). All subsequent procedures were carried out at 4^0C. The homogenate was centrifuged at 100,000 x g for 1 h. The supernatant represented the cytosolic fraction. The pellet was resuspended in buffer A with 0.5% Triton X-100 and sonicated. The mixture was then briefly centrifuged and the supernatant was collected as the membrane fraction. Protein values of each sample were determined by the Bradford method and equal micrograms of proteins were loaded in SDS-polyacrylamide gels.

CHAPTER 3

RESULTS

1. Characterization of cisplatin-resistant cells:

Activation of PKC has been shown to enhance sensitivity of HeLa cells to cisplatin. Therefore, I wanted to investigate if PKC signal transduction is affected during acquisition of cellular resistance to cisplatin. For that purpose, a cisplatin-resistant subline was developed in this laboratory by an *in vitro* selection process.

The initial set of experiments sought to characterize the cisplatin-resistant HeLa (HeLa/CP) cells. Figure 1 shows the cell survival curves of parental and cisplatin-resistant HeLa cells following exposure to different concentration of cisplatin for 24 h. $IC_{50,}$ the concentration of cisplatin required to inhibit cell survival by 50%, was 6.5 uM in HeLa cells, whereas in HeLa/CP cells it could not be determined as it was more than the maximum concentration of the drug used. When cells were incubated with cisplatin for 48 h, the IC_{50} values determined for HeLa and HeLa/CP cells were 0.4 and 6.0 uM respectively (Table 1).

Multiple drug resistance (MDR) is defined as the acquisition of resistance by tumor cells to several structurally and functionally unrelated anticancer drugs

when exposed to a single drug (7). Although cisplatin does not belong to the group of drugs involved in MDR, I examined whether cross-resistance to any other anticancer drugs has developed during acquisition of resistance to cisplatin. Both cell lines were treated with four structurally and functionally different groups of anticancer drugs. The IC_{50} values were determined from the cell survival curve and the ratio of IC_{50} of cisplatin-resistant and -sensitive cells was determined to assess relative resistance to different anti-cancer agents. As shown in Table 1, while HeLa/CP cells were 15-fold resistant to cisplatin, they were only 3.5 and 2.0-fold resistant to cisplatin analogs DACH and carboplatin, respectively. These cells also exhibited only 1.5 and 3.0-fold resistance to doxorubicin and 5-fluorouracil, respectively, and 5-fold resistant to etoposide. It was concluded from these experiments that the developed cell line was highly resistant to cisplatin with a great degree of specificity.

2. Comparison of the expression and regulation of PKC isoforms:

PKC represents a family of 12 closely related isoforms which trigger distinct cellular responses. To examine whether there was a selective alteration in the expression of PKC isoforms in HeLa/CP cells, Western blot analysis was performed with total cellular extracts using isoform-specific antibodies to PKC. Figure 2 shows that both HeLa and HeLa/CP cells expressed PKC-α, $-\delta$, $-\varepsilon$, $-\mu$, $-\zeta$ and $-\iota$. There was a slight decrease in the expression of cPKCα and nPKCε in HeLa/CP cells. In contrast, the expression of nPKCδ was

significantly increased in resistant cells. The abundance of PKCζ, –μ and–ι remained unaltered in the resistant variant. Figure 3 shows the mean densitometric values of three independent sets of the above experiment which confirms my previous result that PKCδ was overexpressed in HeLa/CP cells, whereas PKCα and PKCε expression was slightly decreased.

It has been previously shown that prolonged cellular exposure to PKC activators was necessary for cellular sensitization to cisplatin (13). Therefore, I compared whether PKC activators cause any differential down regulation of PKC isozymes in HeLa and HeLa/CP cells for which I used 3 structurally and functionally distinct group of PKC activators. TPA and PDBu are widely used PKC activators, belonging to the group of tumor promoting phorbol esters. ILV is an analogue of lyngbyatoxin A, and a potent activator of PKC, whereas bryostatin is a non-tumor-promoter and a partial agonist. Western blot analysis was performed from total cellular extracts with isozyme specific antibodies against PKCα, –δ, –ε, –ζ, and –μ. As shown in Figure 4, PKC activators had no effect on the down regulation of aPKCζ in HeLa and HeLa/CP cells. TPA and PDBu caused substantial down regulation of PKCα in both cell lines. All PKC activators caused equivalent down regulation of PKCε in HeLa and HeLa/CP cells. In contrast, while TPA, PDBu, and ILV caused significant down regulation of PKCδ in HeLa cells, they failed to down regulate PKCδ in HeLa/CP cells. A definite shift in mobility was detected in both cell lines following treatment with phorbol esters

and ILV. This was probably due to phosphorylation of PKCδ given phosphorylated proteins have slower mobility in gel electrophoresis. Interestingly, bryostatin 1 induced PKCδ down regulation in both cell types. Thus, a defect in PKCδ down regulation by phorbol esters was associated with cisplatin resistance.

I then compared the time-course of PKCδ down regulation in HeLa and HeLa/CP cells. Cells were exposed to 100 nM TPA for 0-48 h. Western blot analyses with different PKC isozyme-specific antibodies showed (Fig. 5) down regulation of PKCα and PKCε over time in both cell lines. The primary difference observed was in PKCδ down regulation. After 48 h of exposure, TPA caused almost complete down regulation of PKCδ in HeLa cells, whereas in HeLa/CP cells it had little effect. These results suggest that the development of resistance altered the responsiveness of nPKCδ to the tumor promoter TPA.

PKC is usually cytosolic, but PKC activators induce a rapid translocation of PKC from the cytosol to the membrane fraction and prolonged membrane association results in depletion or down regulation of the enzyme. After observing an incomplete down regulation of PKCδ with TPA in HeLa/CP cells, I examined whether TPA-induced translocation was defective in the resistant cells. As shown in Figure 6, PKCα was primarily cytosolic in HeLa and HeLa/CP cells. A 6 h exposure to TPA caused translocation of PKCα to the particulate fraction in both cell lines followed by its down regulation by 24 h. PKCε was mainly membrane

bound. PKCζ was distributed both in the cytosolic and membrane fraction and TPA had no effect on translocation of PKCζ in either cell line. The distribution of PKCδ in cytosolic and particulate fraction and TPA-induced translocation was identical in HeLa and HeLa/CP cells. However, upon prolonged exposure to TPA, PKCδ was depleted from the membrane fraction in HeLa cells, but not in HeLa/CP cells. This result suggests that the deregulation in PKCδ down regulation was not associated with an inability of TPA to induce translocation of PKCδ in HeLa/CP cells.

3. Effects of PKC modulators on cellular sensitivity to cisplatin:

It has been previously shown that PKC modulators influence cisplatin sensitivity in HeLa cells. Figure 7 shows that 1 uM PDBu increased the sensitivity of the parental as well as the resistant cells to cisplatin. In HeLa cells the IC_{50} of cisplatin was 6.4 uM without PDBu and 1.9 uM with PDBu. Therefore, it was decreased by 3-fold with PDBu treatment. In resistant cells in the absence of PDBu, the IC_{50} could not be determined as it was >30 uM, but in the presence of the activator it was 16.8 uM, clearly indicating an enhancement in cisplatin sensitivity by PDBu.

Figure 8 demonstrates a similar enhancement in cisplatin sensitivity with bryostatin 1 in both parental and resistant cell lines. Exposure of HeLa cells with 1 nM bryostatin lowered the IC_{50} of cisplatin from 6.4 uM to 1.7 uM. In HeLa/CP cells the IC_{50} could not be determined in the absence of the activator, but with the

activator 50% cells died with 14 uM cisplatin. It has been shown before that bryostatin 1 exhibits a biphasic concentration response in sensitizing HeLa cells to cisplatin. Therefore, to determine the concentration response of bryostatin 1 on the sensitivity of HeLa/CP cells to cisplatin, cells were treated with different concentrations of bryostatin 1 and percentage of cell survival was determined by the MTT assay. Figure 9 shows that, a biphasic response in cisplatin sensitivity was observed in both cell lines in which maximum sensitization was achieved by 1 nM bryostatin. Yet higher concentrations of bryostatin antagonized its own effect.

To determine whether an alteration in PKC isozyme expression can explain the biphasic effect of bryostatin 1 on cisplatin sensitivity, I examined the effect of different concentrations of bryostatin 1 on PKC isoform expression. As shown in Figure 10, PKCα was gradually depleted with increasing concentrations of bryostatin 1. PKCμ remained unaltered, whereas bryostatin 1 induced a biphasic down regulation of nPKCδ in both cisplatin sensitive and resistant cells. 1 nM bryostatin caused maximum down regulation of PKCδ which coincided with the maximum sensitizing concentration. With higher doses of bryostatin 1, a gradual restoration of PKCδ was noticed. Thus, biphasic down regulation of PKCδ by bryostatin 1 correlated with its biphasic response on cellular sensitization to cisplatin.

CHAPTER 4

DISCUSSION

Acquisition of resistance by tumor cells to cisplatin is the most significant problem in cisplatin therapy. An understanding of the molecular mechanism(s) of cisplatin resistance is essential to develop any improved therapeutic strategy to overcome the problem. The PKC signal transduction pathway has been implicated in influencing cellular sensitivity to cisplatin. The results of the present study demonstrated that, a deregulation in the PKC signal transduction pathway was associated with cellular resistance to cisplatin. Furthermore, PKC activators could reverse cisplatin resistance in the drug-resistant phenotype.

Since PKC plays a critical role in the growth factor signal transduction pathway, it may be detrimental to normal cells to target the PKC signal transduction pathway. The existence of multiple PKC isoforms with differences in structure, enzymatic properties, and intracellular localization strongly suggests specific functions of each PKC isoform (15). Therefore, it is important to characterize different PKC isoforms in the drug resistant phenotype, as little is known about the PKC isoform(s) that is deregulated during development of resistance to cisplatin. The examination of the expression pattern of PKC isoforms revealed a

modest decrease in cPKCα and nPKCε and an increase in the expression of nPKCδ in the drug-resistant HeLa cells. Previous studies have suggested the involvement of PKCδ in the regulation of cell growth and differentiation, apoptosis, and tumor development (16). Therefore, the possibility of PKCδ over expression contributing to drug resistance cannot be ruled out.

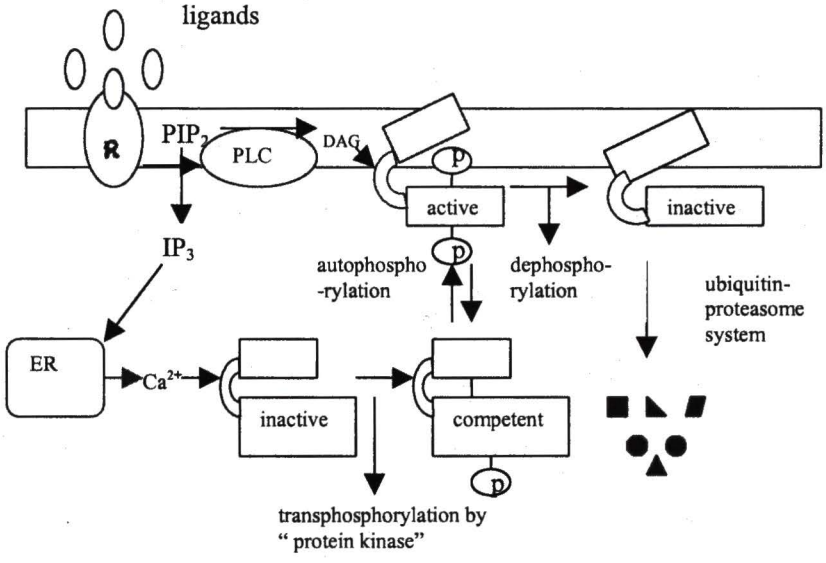
It is generally believed that activation of a family of proteases or caspases is essential for cell death by apoptotic stimuli. PKCδ is a substrate for caspase-3 and proteolytic activation of PKCδ has been directly associated with cell death. A direct correlation between down regulation of PKCδ by bryostatin 1 and cellular sensitivity to cisplatin was observed in the present study. Bryostatin 1 is already in phase I clinical trial and an understanding of how it regulates cisplatin sensitivity has significant clinical potential. Bryostatin 1 was shown to be a more potent activator of PKC than phorbol esters in regulating cisplatin sensitivity. A biphasic effect in the dose-response curve was observed with bryostatin 1 in which 1 nM caused maximum sensitization to cisplatin in both sensitive and resistant cells, whereas higher concentrations of bryostatin protected the cells from the cytotoxic effects of cisplatin. The same biphasic concentration-response was observed for PKCδ down regulation in both cell lines. The maximum sensitizing concentration caused maximum down regulation of PKCδ implicating it as a key player in regulation of cisplatin sensitivity. The unique biphasic dose-response seen with bryostatin may be explained by three possible mechanisms (17). First, PKC possesses two phorbol ester binding domains which appear to

bind with high and low affinity, respectively. Occupancy of the low affinity site and the nature of the ligand at that site probably controls susceptibility to down regulation. Secondly, the high affinity and slow release of bryostatin I at higher concentrations may drive PKC to a cellular sub-compartment where it is protected from degradative enzymes (17). Lastly, since dephosphorylation of activated PKC contributes to down regulation (18), prevention of PKCδ down regulation by increased concentrations of bryostatin 1 may be due to phosphorylation by some other low affinity targets of bryostatin 1 as indirect evidence suggests a low affinity target for bryostatin 1 which leads to enhanced phosphorylation of two 70-kDa proteins and PKCδ might be a target of this pathway (17).

A differential regulation of PKCδ by phorbol esters/ILV and bryostatin 1 was observed in cisplatin sensitive and resistant HeLa cells. ILV and phorbol ester-induced down regulation of PKCδ was incomplete in HeLa/CP cells, whereas in both cell lines bryostatin 1 caused depletion of PKCδ with equal efficacy. This phenomenon can possibly be explained by a previously published report that PKC possesses two phorbol ester binding domains, C1a and C1b, binding to which leads to enzyme activation (20). Different ligands show different selectivity for these domains. For example, phorbol esters and indole alkaloids were selectively dependent on the C1b domain, whereas bryostatin 1 did not show any such selectivity (20). Mutation in the C1b domain affected phorbol ester-induced translocation of PKCδ, whereas C1a domain mutation had little effect on

translocation; but occupancy of the low affinity binding site (C1a) was essential for down regulation (16,17). Data presented here showed that TPA caused identical translocation of PKCδ in both cell lines, but down regulation of the isozyme from the membrane fraction was altered in the resistant cells. This finding provoked us to speculate an alteration or mutation in the C1a binding domain of PKC to be associated with cisplatin resistance.

It has been hypothesized that transphosphorylation of the nascent protein



(Hypothetical pathway of PKC activation, translocation, dephosphorylation, and degradation)

by PKC kinase converts PKC to a competent cytosolic form, which then translocates to the plasma membrane. Here it phosphorylates itself upon activation by DAG, TPA, or bryostatin, and phosphatidylserine (18). This is followed by dephosphorylation which inactivates the enzyme and results in its degradation or down regulation by the ubiquitin-proteasome pathway or by other

proteases like caspases. Ubiquitination is apparently isoform specific, and the activation of one PKC isoform does not stimulate ubiquitination and down regulation of other inactive isoforms (22). We demonstrated that, TPA-induced translocation of PKCδ was unaffected in the cisplatin-resistant HeLa cells, but down regulation was altered. However, we also showed an identical down regulation of PKCα and PKCε in both cell lines which excluded the possibility of any alteration in the degradation pathway. Rather, it supported our previous speculation of a probable alteration in the phorbol ester binding domain of PKCδ in HeLa/CP cells. As the down regulation is effected through degradation, and without a shutdown of synthesis, a steady state level of PKC is maintained even in the down regulated state (21). Our data showed that a substantial amount of PKCδ remained in the HeLa/CP cells after 48 h of exposure to TPA, whereas in HeLa cells the isoform was almost completely depleted, showing only a very faint band which reflected the steady state level of the enzyme. Therefore, it was evident that the down regulation of PKCδ was defective in the cisplatin resistant cells.

Future studies should be directed towards a better understanding of the mechanisms by which PKC affects cisplatin resistance. Overexpression of nPKCδ and site-directed mutagenesis at different phosphorylation and substrate binding sites of the isozyme in cell culture models would clarify the mechanistic aspect of the differential regulation of PKCδ by PKC activators and would also enable the future researcher to further understand whether altered PKC signal

transduction is causal to or an effect of acquisition of resistance. Nevertheless, the observations that PKC activators could enhance sensitivity of cisplatin-resistant HeLa cells to cisplatin suggest that PKCδ could provide an important target to circumvent cisplatin resistance.

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FIGURE LEGENDS

Figure 1.Comparison of cisplatin sensitivity in HeLa and HeLa/CP cells. HeLa (○) and HeLa/CP (Δ) cells were plated in 96-well plates. After 48 h they were exposed to varying concentration of cisplatin for 24 h. The percentage of cell survival was determined by the MTT assay as described in "Methods". Each symbol represents mean values from 3 independent set of experiments with standard error (S.E.).

Figure 2. Comparison of PKC isozyme expression in HeLa and HeLa/CP cells. Total cellular extracts were obtained and western blot analyses were performed with isozyme specific antibodies to PKCα, –δ, –ε, –μ, –ι, and–ζ as described in "Methods". Arrows indicate immuno-reactive bands.

Figure 3. Comparison of PKC isozyme content in HeLa and HeLa/CP cells. The abundance of PKC isoforms was quantified by scanning Western blots with a laser densitometer. The bars show mean values with S.E., solid bars represent HeLa cells, and hatched bars HeLa/CP cells. Statistical significance: * $p > 0.05$, ** p and *** $p \leq 0.01$.

Figure 4. Effect of PKC activators on the down regulation of PKC isozymes in HeLa and HeLa/CP cells. Cells were treated with TPA (100 nM) , PDBu (1 uM), bryostatin (1 nM), and ILV (10 uM) for 24 h. Total protein was then extracted and western blot analyses were performed with PKC isozyme specific antibodies. The same blot was stripped and incubated with different antibodies.

Figure 5. Comparison of PKC down regulation by TPA in HeLa and HeLa/CP cells. Cells were exposed to TPA for the indicated periods of time and Western blot analyses were performed as described in "Methods". As caspase 2 remains unaltered by treatment with TPA in HeLa cells, it was used to check equivalent loading. In each case arrows indicated immuno-reactive bands.

Figure 6. Comparison of PKC translocation By TPA in HeLa and HeLa/CP cells. Cells were treated with TPA (100 nM) for 0-48 h. Cytosol and membrane fractions were separated as described in "Methods" and Western blot analyses were performed with PKC isozyme-specific antibodies. Caspase 2 was used as a cytosolic marker and COX II was used as membrane marker (data not shown).

Figure 7. Comparison of the effect of PDBu on the sensitivity of HeLa and HeLa/CP cells to cisplatin. Cells were plated in 96-well plates and after 48 h HeLa (*circles*) and HeLa/CP (*triangles*) were treated with (*closed symbols*) or w

ithout (*open symbols*) 1 uM PDBu for 24 h. Cells were then treated with varying concentrations of cisplatin for another 24 h. The cell survival was determined by an MTT assay as described in "Methods". Each symbol represents mean value of three independent set of experiments with S.E.

Figure 8. Comparison of the effect of bryostatin I on the sensitivity of HeLa and HeLa/CP cells to cisplatin. Cells were first plated in 96-well plates. After 48 h, HeLa cells (*circles*) and HeLa/CP cells (*triangles*) were treated either with (*closed symbols*) or without (*open symbols*) 1 nM bryostatin for 24 hours. They were then treated with varying concentrations of cisplatin for additional 24 h. Each symbol represents the mean value of three independent experiments with S.E.

Figure 9. Effect of different concentrations of bryostatin I on the sensitivity of HeLa and HeLa/CP cells to cisplatin. Cells were pre-treated with different concentrations of bryostatin for 24h. The cells were then exposed to varying concentrations of cisplatin for 24 h. Cell survival was determined by an MTT assay. The bar graph shows mean IC_{50} values from two separate experiments in HeLa/CP cells. The experiment was done once with HeLa cells for comparison, but repetition was not necessary as the data has already been published (14). Statistical significance: $* p < 0.05$.

Figure 10. Effect of different concentrations of bryostatin I on PKC down regulation in HeLa and HeLa/CP cells. Both cell lines were exposed to different concentrations of bryostatin I for 24 h. Western blot analyses were performed with the total cellular extracts as described in the "Methods." Arrows indicate the immuno-reactive bands.

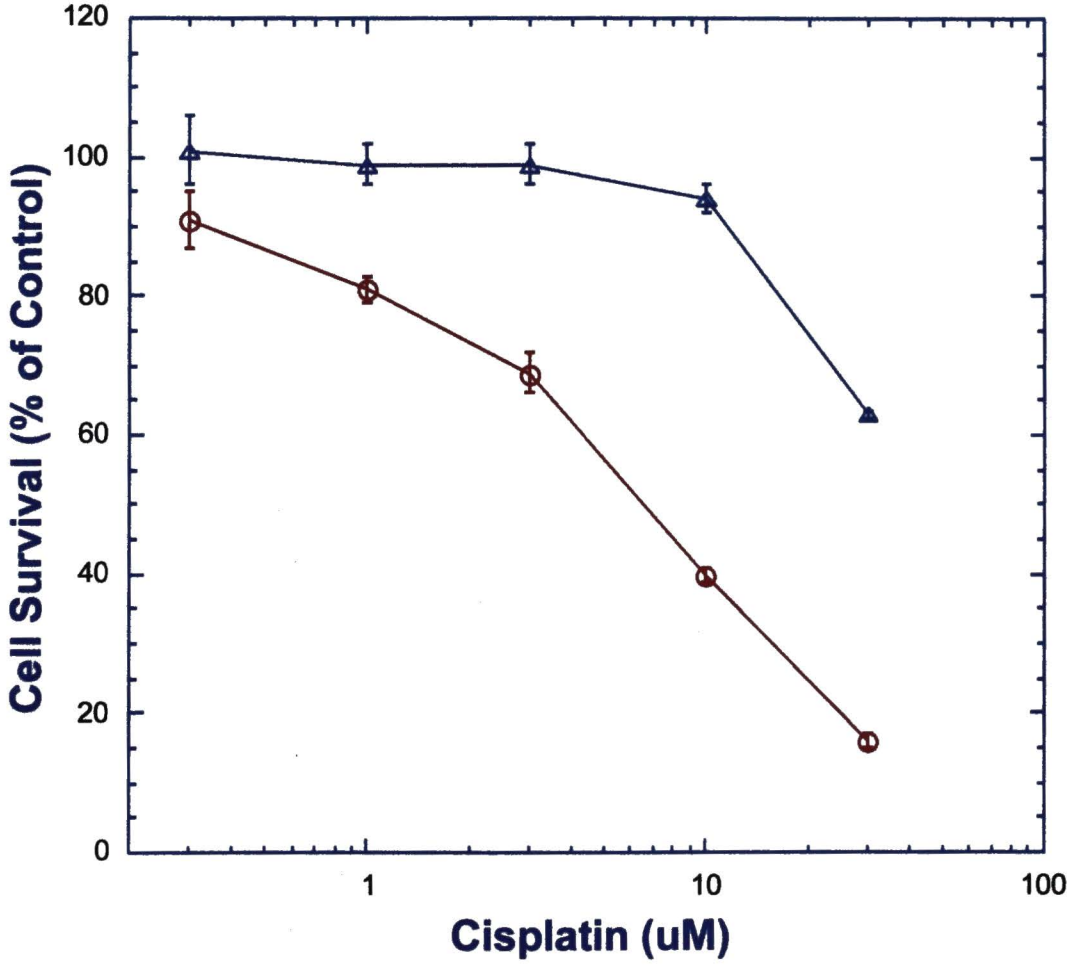

Figure 1: Comparison of cisplatin sensitivity in HeLa and HeLa/CP cells.

Drugs	IC_{50} (uM)		-fold resistance
	HeLa	HeLa/CP	
Cisplatin analogues			
Cisplatin	0.4	6.0	15.0
DACH	4.0	14.4	3.5
Carboplatin	14.0	30	2.0
Doxorubicin	3.5	5.2	1.5
5-Fluorouracil	4.0	12.5	3.0
VP-16/ Etoposide	4.0	20.0	5.0

Table 1 : Effect of different anticancer drugs on the proliferative activity of HeLa and HeLa/CP cells. Cells were treated with different anticancer drugs for 48 h. Cell survival was determined by the MTT assay. The table shows a comparison of the IC50 values of these compounds in cisplatin-sensitive and -resistant cells.

Cisplatin analogues: Resemble bifunctional alkylating agents and form intrastran crosslinks with DNA.

Doxorubicin: Binds tightly to DNA by its ability to intercalate between base pairs and causes DNA strand breaks.

5-Fluorouracil: Purine analogue. Selectively toxic to proliferating cells.The active metabolite inhibits DNA synthesis

Etoposide: Forms a complex with the enzyme Topoisomerase II which results in a single strand breakage of DNA.

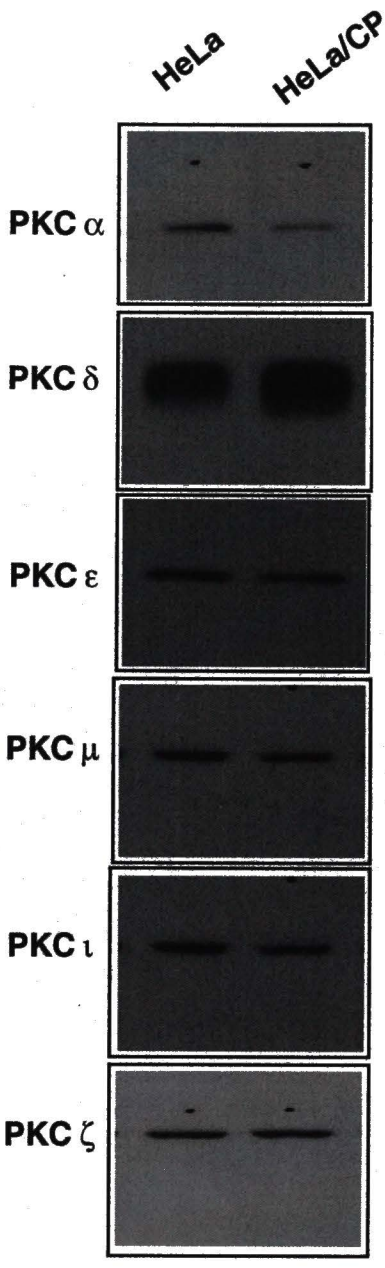

Figure 2 : Comparison of PKC isozyme expression in HeLa and HeLa/CP cells.

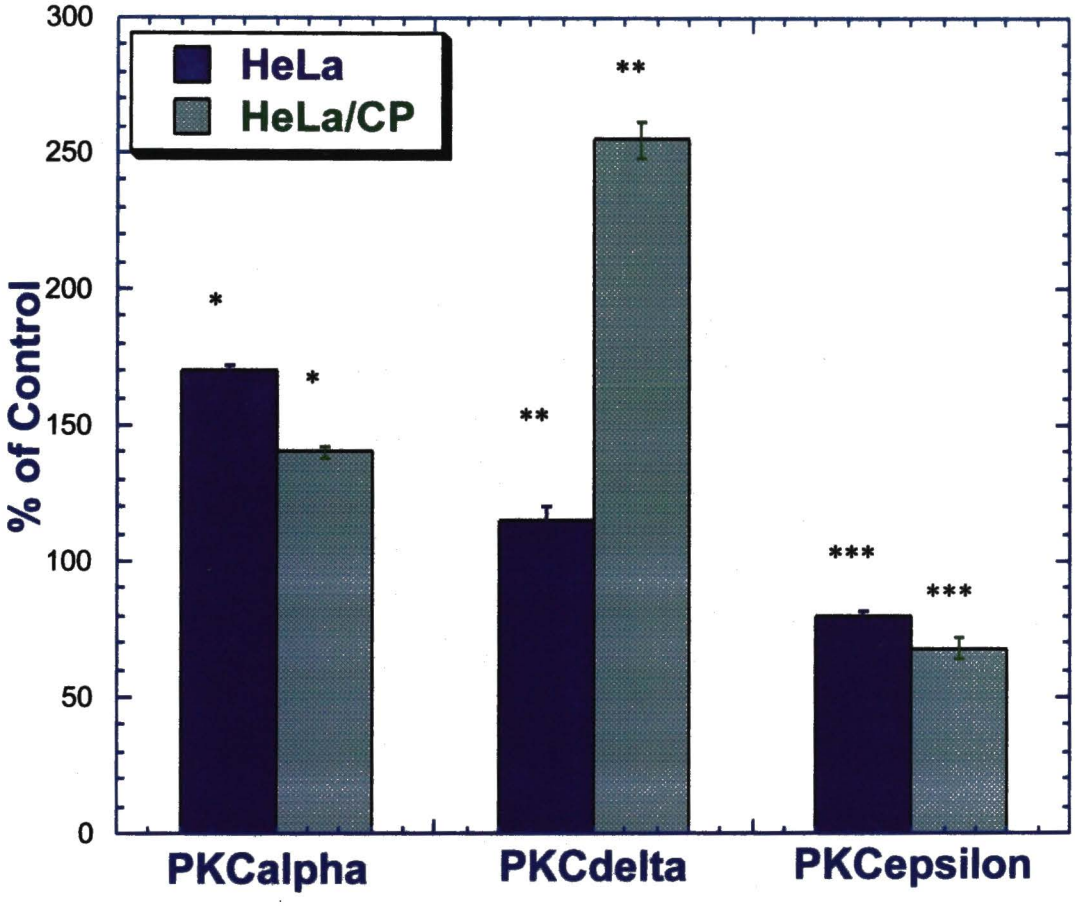

Figure 3: Comparison of PKC isozyme content in HeLa and HeLa/CP cells

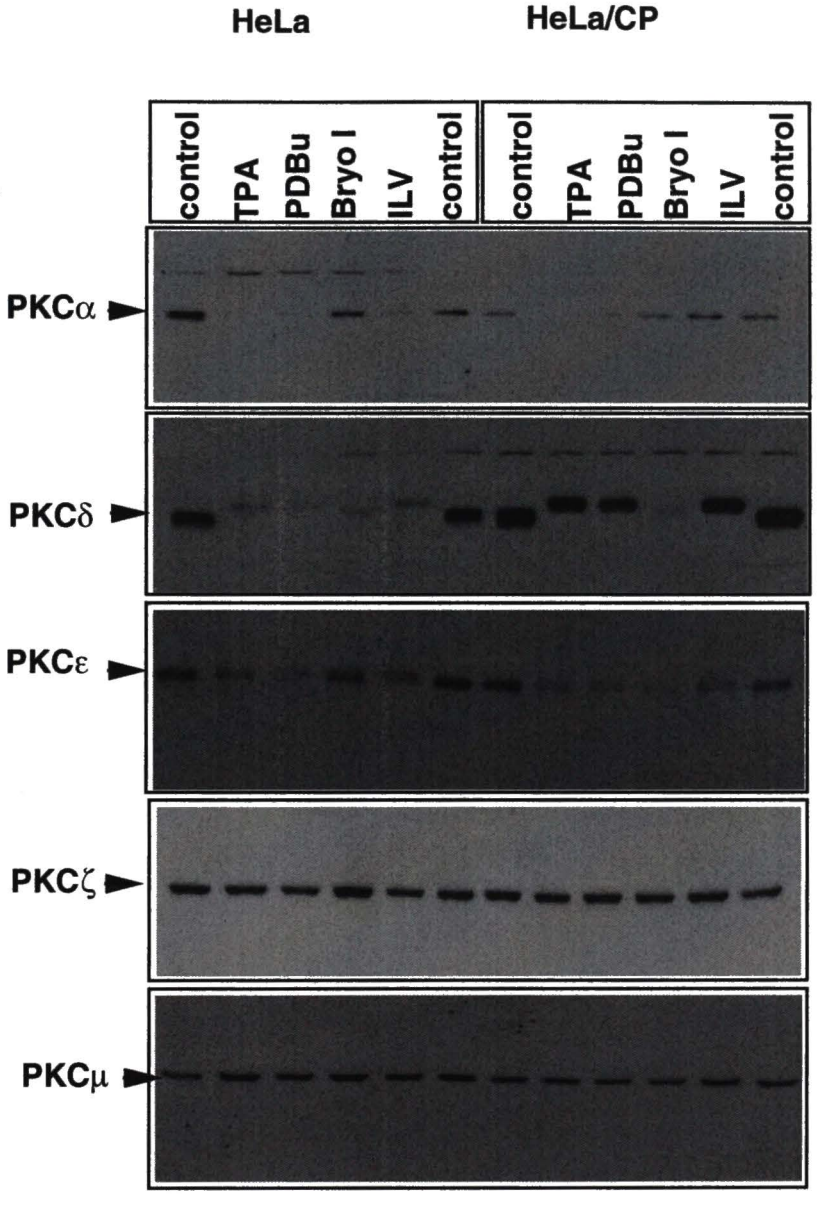

Figure 4 : Effect of PKC activators on the down-regulation of PKC isozymes in HeLa and HeLa/CP cells.

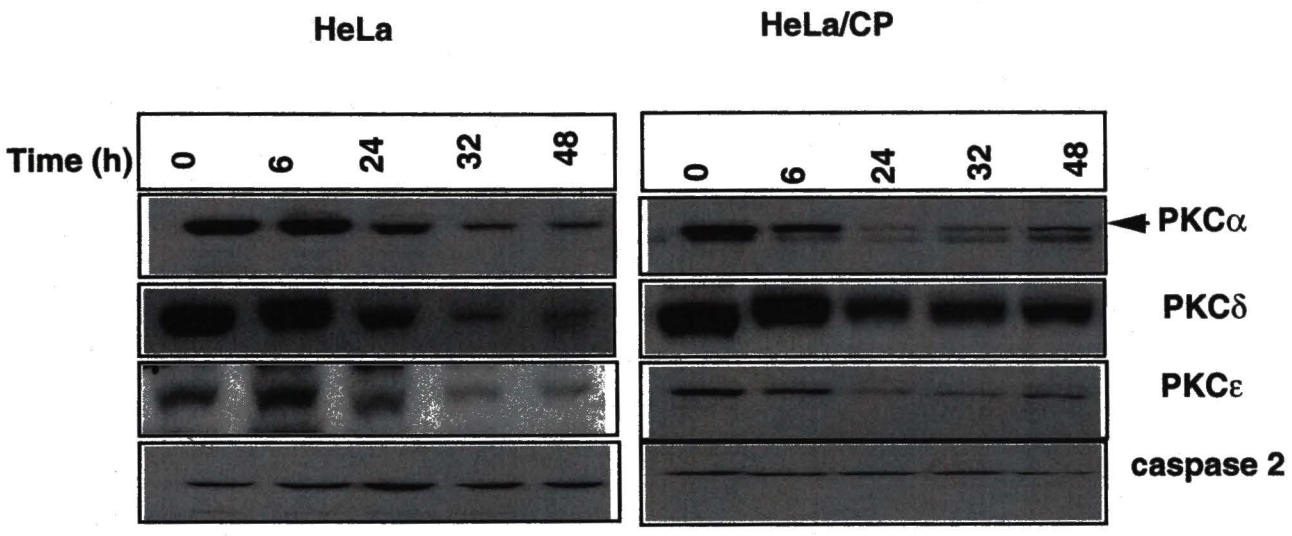

Figure 5 : Comparison of PKC down regulation by TPA in HeLa and HeLa/CP cells

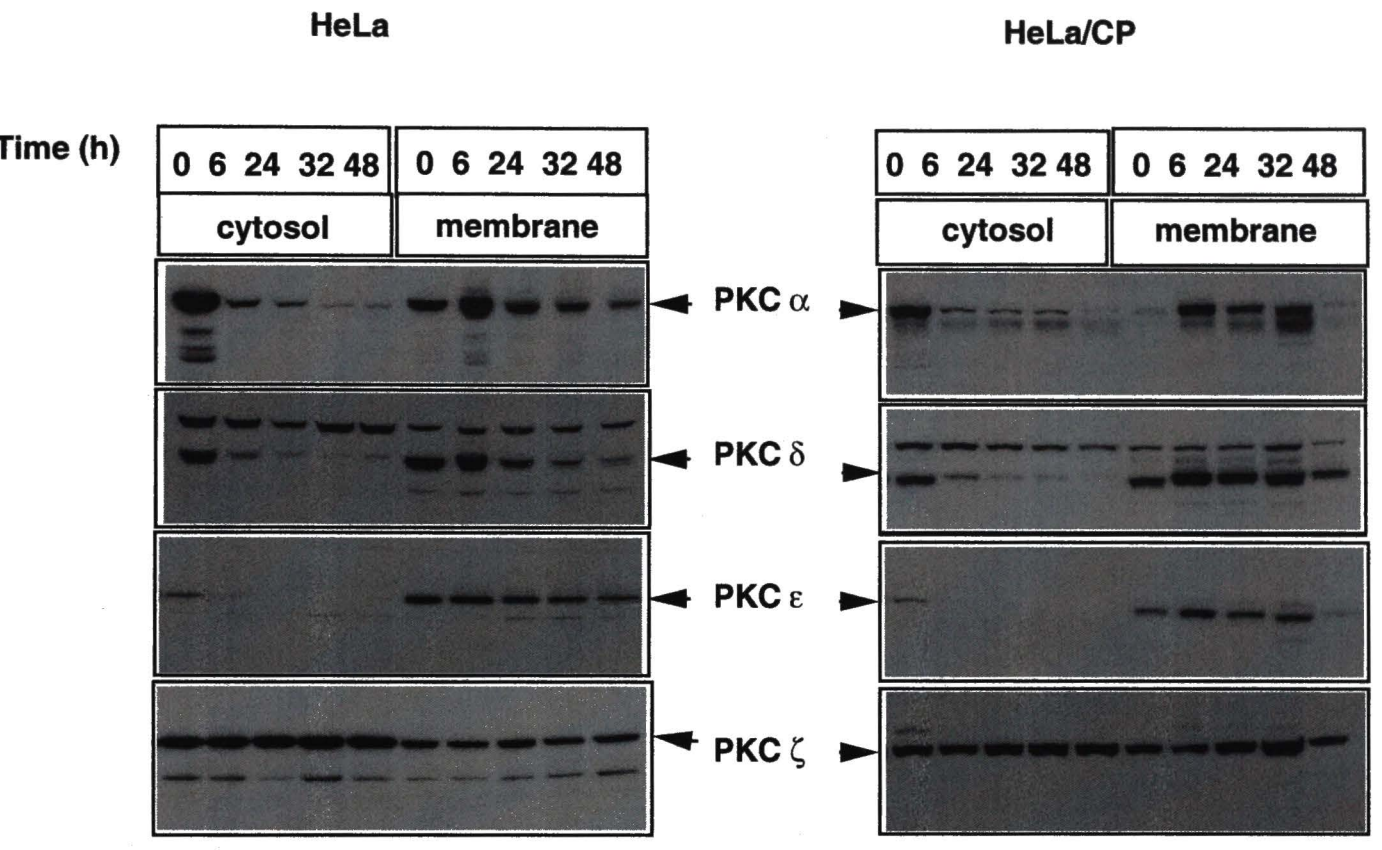

Figure 6 :Comparison of PKC translocation by TPA in HeLa and HeLa/CP cells.

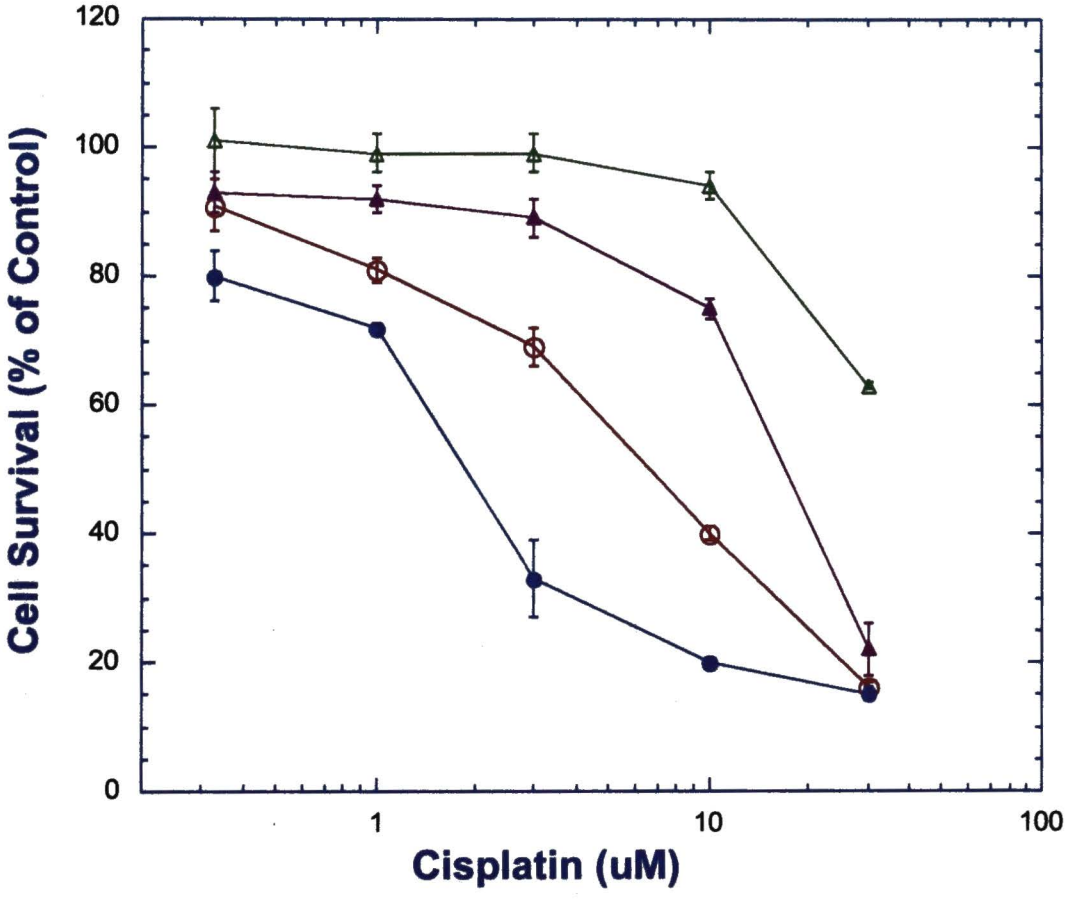

Figure 7: Comparison of the effect of PDBu on the sensitivity of HeLa and HeLa/CP cells to cisplatin.

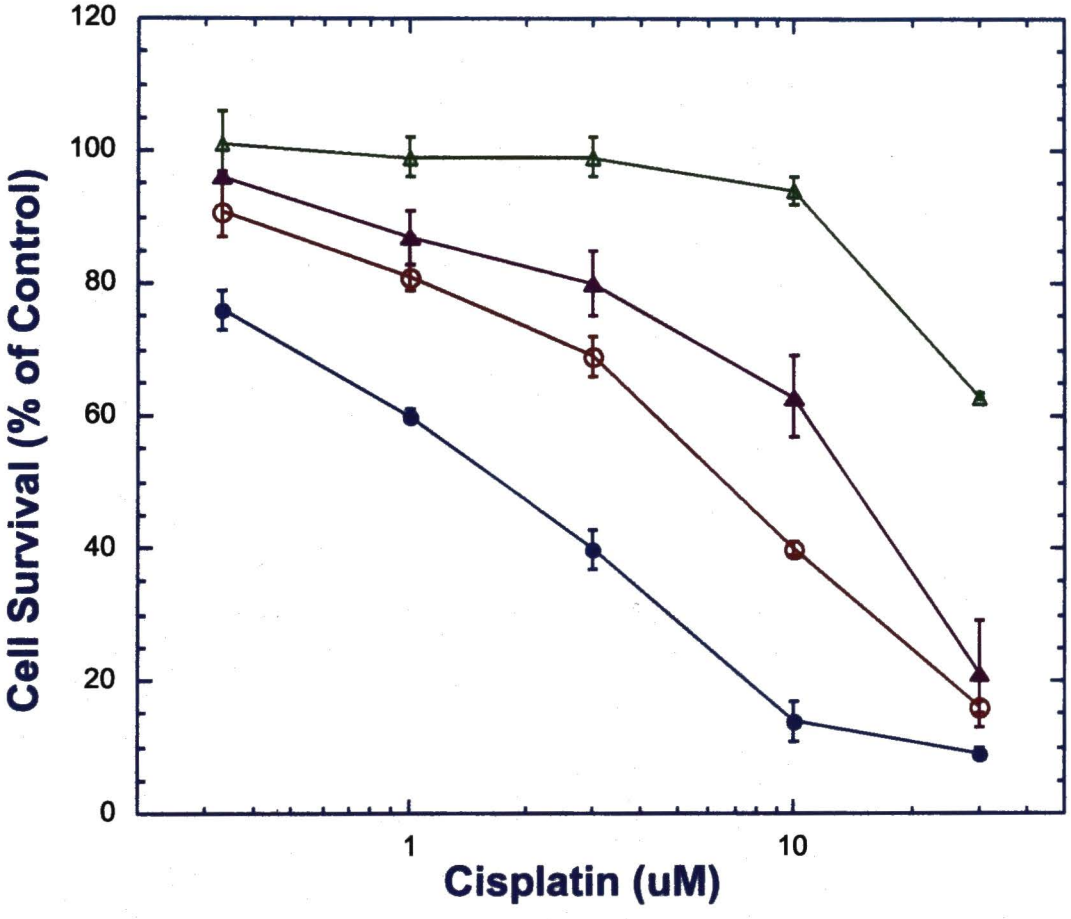

Figure 8 : Comparison of the effect of bryostatin I on the sensitivity of HeLa and HeLa/CP cells to cisplatin.

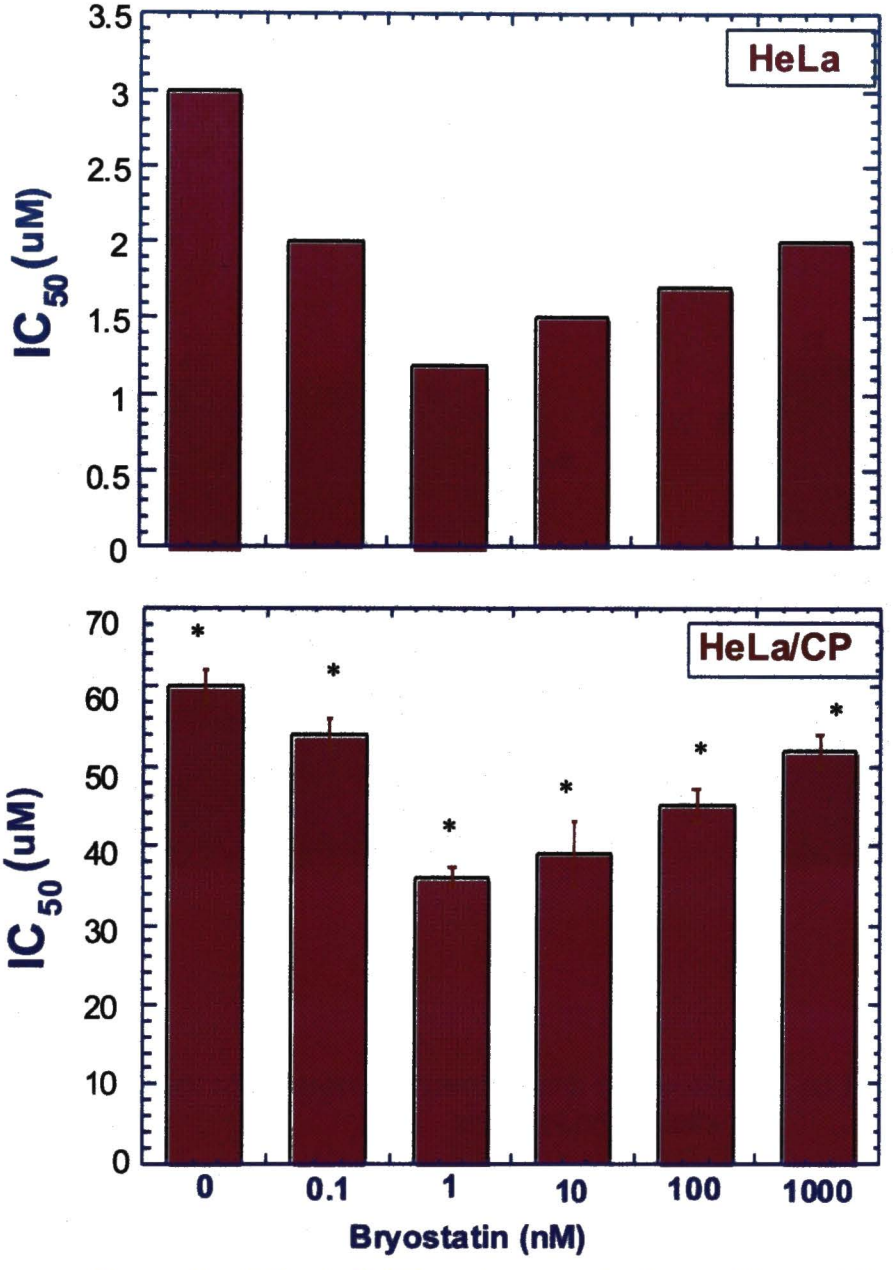

Figure 9 : Effect of different concentrations of bryostatin I on the sensitivity of HeLa and HeLa/CP cells to cisplatin.

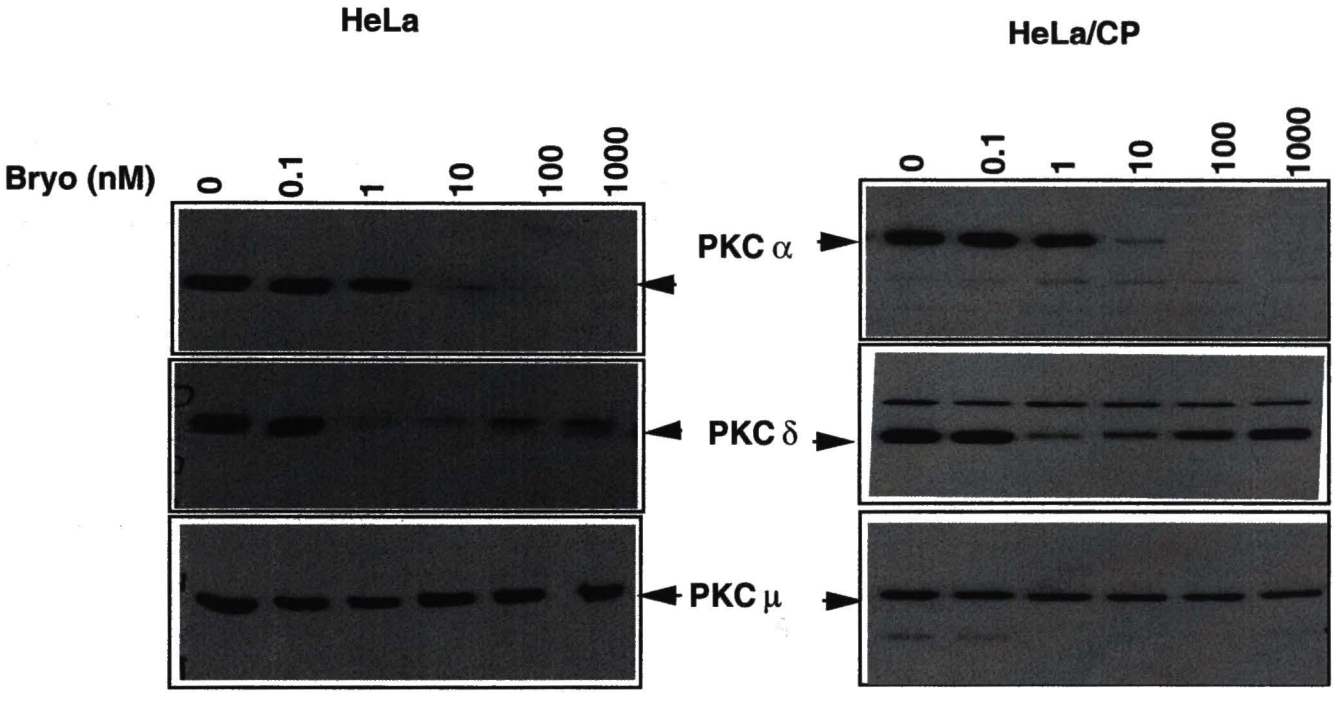

Figure 10 : Effect of different concentrations of bryostatin I on PKC down regulation in HeLa and HeLa/CP cells