Elevated Level of Nuclear Protein Kinase C in Multidrug-resistant MCF-7 Human Breast Carcinoma Cells

Sung Ae Lee, James W. Karaszkiewicz, and Wayne B. Anderson

ABSTRACT

Previous studies have demonstrated elevated levels of protein kinase C (PKC) activity in multidrug-resistant human breast carcinoma MCF-7/ADR cells compared to control drug-sensitive MCF-7/WT cells (R. L. Fine, J. Patel, and B. A. Chabner, Proc. Natl. Acad. Sci. USA, 85: 582-586, 1988). In our present studies, immunohistochemical localization analysis using a polyclonal PKC antibody recognizing the α, β, and γ subtypes of PKC demonstrates that immunoreactivity is enhanced in MCF-7/ADR cells, with pronounced staining noted in the nuclear region. Other studies with purified nuclei isolated from MCF-7/ADR cells also show a marked increase in the intensity of immunostaining for PKC when compared to nuclei prepared from control MCF-7/WT cells. Western blot analysis of proteins extracted from purified nuclei preparations further establishes an increase in PKC enzyme protein associated with the nuclear fraction of MCF-7/ADR cells. Subcellular fractionation studies also indicate that MCF-7/ADR cells have 4-8 times higher nuclear PKC activity compared to that of control MCF-7/WT cells. MCF-7/ADR cells also possess 3-5-fold elevated cytosolic PKC activity, while a <2-fold increase is found in PKC activity associated with the plasma membrane fraction of MCF-7/ADR cells. Examination of these extracts with PKC isotype-specific antisera, as well as by DEAE-cellulose chromatography, reveals that nuclei prepared from MCF-7/ADR cells contain markedly elevated amounts of a slightly altered form of PKCa. These results suggest that elevated levels of a modified form of PKCa at the nucleus may play a role in modulating nuclear events to promote the development of multidrug resistance in MCF-7 cells.

INTRODUCTION

Development of resistance to cancer chemotherapeutic agents such as doxorubicin (Adriamycin) and vinblastine severely limits the use of these drugs in cancer treatment. A particular phenotype of resistance, called multidrug-resistance (MDR), exhibits a broad spectrum of resistance to anticancer drugs derived from natural products or their derivatives including anthracyclines, Vinca alkaloids, epipodophyllotoxins, and actinomycin D (1, 2). Although the mechanism associated with the development of multidrug resistance is still unclear, a common feature of multidrug resistance is a net decrease in the intracellular accumulation of drugs as a consequence of enhanced drug efflux and the overexpression of a membrane-associated P-glycoprotein (3-7). However, several lines of evidence suggest that mechanisms in addition to a net decrease in drug accumulation may be also involved in modulating multidrug resistance. Among other possible contributing mechanisms are (a) alterations in glutathione metabolism (8-11), (b) a decrease in metabolic activation of drug (12), and (c) differential oxygen free-radical susceptibility (13). It also has been reported that PKC activity is increased in several cell types exhibiting the MDR phenotype, including Adriamycin-resistant MCF-7 human breast carcinoma cells (14). The enhanced levels of PKC in MCF-7 cells resistant to Adriamycin and the modulation of the drug-resistance phenotype by agents which alter PKC activity (i.e., TPA, which first activates and then subsequently down-regulates PKC, as well as H-7 [1-(5-isouquinolinesulfonyl)-2-methylpiperezine] PKC inhibitor) suggest a role for PKC in multidrug resistance (14-17). Thus, it is of importance to characterize the biological and regulatory properties of PKC found elevated in multiple drug-resistant cell types.

To better define a possible role of PKC in the modulation of the multidrug resistance phenotype, we have assessed changes in PKC activity, subcellular distribution, and isotype pattern in drug-sensitive MCF-7/WT cells and in drug-resistant MCF-7/ADR cells. The MCF-7/ADR cells were found to possess markedly elevated levels of a modified form of PKCa in the nucleus. These results suggest that an increase in a specific form of PKCa in the nucleus of MCF-7 human breast carcinoma cells may play a role in modulating nuclear events such as altered transcription to promote the development of multidrug resistance in MCF-7 cells.
PKC Antibodies. Four different polyclonal PKC antibodies were used in these studies. Antibodies were raised in rabbits against keyhole limpet hemocyanin-conjugated synthetic peptides corresponding (a) to the NH₂-terminal pseudosubstrate region common to isotypes α, β, and γ (FARKGALRQKNV) and thus pan-specific for these isotypes, (b) to the iso type-specific hinge region peptide (KLPGAGNYKVPSEPD), (c) to the β iso type-specific COOH-terminal region (SEFEQFSFVNS-EFLKPEVKS), and (d) to the γ iso type-specific COOH-terminal region (AEFQFQTYVNDPDVFH) of PKC. The isotype specificity was screened using extracts prepared from COS-7 cells transfected with the α, β, and γ iso types of PKC. Each antiserum was found to be specific for the PKC isotype indicated and exhibited no cross-reactivity with the other PKC iso types tested.

Immunohistochemical Localization of PKC. MCF-7/WT cells and MCF-7/ADR cells grown on Lab-Tek Permanox tissue culture chamber slides were washed with PBS, fixed with 4% formaldehyde/0.05% glutaraldehyde in PBS, and then permeabilized with 0.1% Triton X-100 in PBS. The permeabilized cells were sequentially incubated with 10% normal serum, 10% normal goat serum, and 2% BSA (globulin-free) in PBS for 30 min each to block nonspecific IgG binding. Then, the cells were incubated overnight at 4°C with PKC antiserum (pan-specific for α, β, and γ isotypes of PKC) in PBS containing 2% normal goat serum, 1% BSA (globulin-free), and 0.05% Triton X-100. The cells then were rinsed with PBS and subsequently incubated with biotinylated goat anti-rabbit IgG in PBS containing 1% BSA (globulin-free) and 0.01% Tween 20. The immunoreactivity to PKC was localized by incubation with alkaline phosphatase-conjugated avidin D and subsequent color development by incubation with Vector ABC kit (Vector Laboratories, Burlingame, CA).

Preparation of Nuclear-Cytoskeletal Components. Intact MCF-7/WT cells and MCF-7/ADR cells grown on Lab-Tek Permanox tissue culture chamber slides were rapidly washed with warm (30°C) PBS and extracted for 10–14 min at 30°C by incubation with buffer solution containing 0.1 M piperazine-N,N′-bis(2-ethanesulfonic acid) (pH 6.9), 5 mM MgCl₂, 0.2 mM EGTA, 4 mM glycerol, 100 μg/ml leupeptin, and 0.1% Triton X-100. The solubilized fraction was quickly removed to leave nuclear-cytoskeletal structures attached to the substratum. The remaining nuclei and cytoskeletal structures were washed twice with cold PBS and subsequently processed for the immunohistochemical localization of PKC as described above.

Subcellular Fractionation. Cells were washed twice with ice-cold Ca²⁺- and Mg²⁺-free D-PBS and then harvested by scraping from culture dishes into ice-cold Equilibrium Buffer (20 mM Tris-HCl (pH 7.5) containing 2 mM EDTA, 2 mM EGTA, 2 mM dithiothreitol, and protease inhibitors (100 μg/ml leupeptin, 50 μg/ml aprotinin, 50 μg/ml 4-amidinophenyl-methanesulfonyl fluoride, 10 μg/ml soybean trypsin inhibitor, and 100 ng/ml pepstatin A), and then disrupted by 100 strokes (pestle B) with a Dounce homogenizer. Crude nuclei were isolated by centrifugation of total cell homogenate at 1000 × g for 5 min. This crude nuclear pellet was subsequently purified by sucrose density centrifugation as described previously (18). The purity of the nuclear preparations was determined by electron microscopy and was routinely monitored by phase contrast microscopy after staining with methylene blue. The plasma membrane-enriched fraction was prepared by centrifugation of the 100,000 × g supernatant at 100,000 × g for 20 min. The cytosolic fraction was isolated by centrifugation of the 100,000 × g supernatant at 100,000 × g for 60 min. For PKC activity measurement the total cell homogenate, purified nuclear fraction, and plasma membrane-rich fractions were solubilized with 1% (w/v) CHAPS on ice overnight.

Assay of PKC Activity. The reaction was initiated by addition of 1–3 μg of protein sample to a reaction mixture containing 20 mM Tris-Cl (pH 7.5), 1 mM CaCl₂, 10 mM MgCl₂, 100 μM [γ-Th²²]ATP (3 × 10⁴ cpm), 2 mM dithiothreitol, and 200 μg/ml histone type III-S or 4 μM PKC substrate peptide [(Ser²²)PKC(19–31)] ( Gibco) as phosphoacceptor substrate in a total volume of 100 μl. The final concentration of EDTA and EGTA in the reaction mixture contributed by the protein sample was 0.4 mM for each. Phosphatidylserine (25 μg) and diolein (2.5 μg) were added to some tubes in a volume of 20 μl to demonstrate phospholipid-dependent protein kinase activity. After incubation for 6–10 min at 30°C, 50 μl of aliquots of the reaction mixture were spotted onto paper strips (Whatman P-81 ion exchange chromatography paper). All strips were then immersed in 100 μl of 0.3 M phosphoric acid and air dried, and radioactivity retained on the papers was determined. Protein kinase C activity was calculated from the difference in ³²P incorporated into histone in the presence and absence of added phospholipids and calcium and was expressed as ³²P incorporated/min/mg protein.

Colorimetric MTT Test for Cytotoxicity to Adriamycin. MCF-7 cells were seeded in 24-well plates (Costar, Cambridge, MA) at a density of approximately 25,000 cells/well in IMEM as described above. After 24 h the medium was removed and 1-ml aliquots of IMEM containing various concentrations of doxorubicin and supplemented with 2 mM l-glutamine, 2 mg/ml l-proline, 50 μg/ml gentamicin and 10% heat-inactivated fetal bovine serum were added to each well. After 48 h of incubation at 37°C, 100 μl of MTT at a concentration of 5 mg/ml in D-PBS were added to each well, and the cultures were further incubated for 2 h at 37°C. The reaction was terminated by the addition of the medium along with the MTT dye, and the formazan precipitate formed was dissolved in 1 ml of dimethyl sulfoxide. The relative number of viable cells remaining after Adriamycin treatment was estimated by the change in absorbance at 590 nm. The relative percentage of growing cells was calculated as Absorbance in wells treated with doxorubicin + Absorbance in control wells without doxorubicin × 100

Western Blot Analysis of PKC. Subconfluent MCF-7 cells grown in IMEM were rapidly washed twice with ice-cold Ca²⁺- and Mg²⁺-free (CM-free) D-PBS. A hot SDS total cell extract then was prepared by immediately scraping the cells into boiling SDS-polyacrylamide gel electrophoresis sample buffer and drawing through a 23-gauge needle 10 times to shear DNA (19). The extracts were then boiled for 5 min. Electrophoresis of the extracted proteins was carried out using SDS-polyacrylamide gel electrophoresis minigels unless otherwise described. The protein bands then were transferred electrophoretically to Immobilon-P membrane (Millipore, Bedford, MA). Following electrotransfer, the membrane was incubated with 2% (w/v) Carnation nonfat dry milk in CM-free D-PBS for 30 min at room temperature to block nonspecific IgG binding sites and then washed with CM-free D-PBS for 10 min. The membrane then was incubated overnight at 4°C with protein kinase C antiserum diluted 1:500 in CM-free D-PBS containing 2% nonfat dry milk. The membrane exposed to antiserum then was washed sequentially with 0.25% Tween 20 in CM-free D-PBS for 10 min, with 0.05% Tween 20 in CM-free D-PBS twice (10 min each wash), and finally with CM-free D-PBS for 10 min at room temperature. After primary antiserum incubation and washings, the membrane was incubated with biotinylated secondary antibody diluted 1:1000 in CM-free D-PBS containing 2% nonfat dry milk for 1.5–2.5 h at room temperature and then washed as described above. The washed membrane then was incubated with alkaline phosphatase-conjugated streptavidin diluted 1:1000 in CM-free D-PBS containing 2% nonfat dry milk for 30 min at room temperature and again washed as described above. PKC was visualized by incubation with 0.4 mM nitroblue tetrazolium-0.4 mM 5-bromo-4-chloro-3-indolyl-phosphate in 100 mM TrisCl (pH 9.5) buffer solution containing 100 mM NaCl and 10 mM MgCl₂ at room temperature for 10–20 min. To terminate the color development reaction the membrane was washed with 50 mM EDTA, subsequently washed with deionized H₂O, and then air dried.

Protein Determination and Data Expression. Protein concentration was determined by Bradford protein assay (20) with BSA as standard. Data are expressed as mean ± SEM. Error bars smaller than the symbols or lines used in the figures have been omitted.

RESULTS

Immunohistochemical Localization of PKC in MCF-7/WT and MCF-7/ADR Cells. The immunohistochemical localization of PKC was determined with rabbit anti-peptide antisem er pan-
ELEVATED NUCLEAR PKC AND DRUG RESISTANCE

specific for the α, β, and γ isotypes of PKC prepared as described in “Materials and Methods.” In intact MCF-7 cells, multidrug-resistant MCF-7/ADR cells showed a markedly enhanced immunoreactivity to PKC compared to that of drug-sensitive MCF-7/WT cells (Fig. 1). The most pronounced difference was found in the nuclear region. A dense immunostaining was found in the nuclear region of MCF-7/ADR cells while little immunostaining was present in the perinuclear region of MCF-7/WT cells. This finding was confirmed in studies using isolated nuclei (Fig. 2). Nuclei isolated from MCF-7/ADR cells showed a marked increase in intensity of immunostaining for PKC compared to nuclei prepared from drug-sensitive MCF-7/WT cells. A similar result was observed with α isotype-specific PKC antibody, while no significant difference in the intensity of immunostaining for β-isotype PKC was found in MCF-7/WT and MCF-7/ADR cells (data not shown).

Subcellular Distribution of PKC Activity in MCF-7/WT Cells and MCF-7/ADR Cells. It has been previously demonstrated that PKC activity is elevated in MCF-7/ADR cells compared to MCF-7/WT cells (14). To better characterize the elevated PKC activity found in MCF-7/ADR cells, the subcellular distribution of PKC activity in MCF-7/WT and MCF-7/ADR cells was compared (Table 1). In the process of subcellular fractionation, the nuclear pellets isolated from MCF-7/WT and MCF-7/ADR cells were further purified as described by Thomas et al. (18). The purity of the nuclear fraction was verified by electron microscopic examination (data not shown). The total cell homogenate of MCF-7/ADR cells exhibited about a 4-fold increase in total PKC activity compared to that of MCF-7/WT cells. The most striking difference in total PKC activity, and also in specific activity, was found in the nuclear fraction. The nuclear fraction isolated from MCF-7/ADR cells exhibited a 4–8-fold higher PKC specific activity when compared to those of MCF-7/WT cells. Similar results were observed when the nuclear preparation was isolated following disruption of the cells in homogenization buffer containing detergent such as 0.05% Triton X-100 or containing 0.5 M hexylene glycol (data not shown). A higher level (3–5-fold) of PKC activity was also found in the cytosolic fraction of MCF-7/ADR cells, while a lesser increase (<2-fold) was noted in plasma membrane-associated PKC activity of MCF-7/ADR cells. To exclude the possibility that the significant pool of nuclear PKC activity found in the MCF-7/ADR cells might originate from residual contamination from the cytosolic pool, we carried out a time course analysis of changes in the distribution and down-regulation of PKC in the different subcellular fractions in response to treatment of these cells with 1 μM TPA (Fig. 3). Exposure of MCF-7/ADR cells to 1 μM TPA resulted...
ELEVATED NUCLEAR PKC AND DRUG RESISTANCE

Fig. 2. Immunohistochemical localization of protein kinase C in the nuclear-cytoskeletal fraction of drug-sensitive MCF-7/WT and multidrug-resistant MCF-7/ADR human breast carcinoma cells. The nuclear-cytoskeletal fraction was prepared as described in “Materials and Methods.” Rabbit anti-peptide antiserum (pan-specific for NH2-terminal pseudosubstrate region of α, β, and γ isotypes of PKC) was used in this study, while normal rabbit serum was used as a control. The immunoreactivity to PKC visualized with a Vector ABC kit was photographed with an Olympus Vanox microscope at × 400.

Table 1  Subcellular distribution of protein kinase C activity in control drug-sensitive MCF-7/WT and multidrug-resistant MCF-7/ADR cells

Subcellular fractionation and PKC activity measurement were carried out as described in “Materials and Methods.” Data are expressed as mean ± SEM.

<table>
<thead>
<tr>
<th>Subcellular fraction</th>
<th>MCF-7/WT</th>
<th>MCF-7/ADR</th>
<th>Fold increase</th>
<th>MCF-7/WT</th>
<th>MCF-7/ADR</th>
<th>Fold increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total homogenate</td>
<td>8.79 ± 1.37</td>
<td>32.51 ± 2.03</td>
<td>3.7</td>
<td>0.25 ± 0.04</td>
<td>1.12 ± 0.07</td>
<td>4.5</td>
</tr>
<tr>
<td>Nucleus</td>
<td>0.78 ± 0.08</td>
<td>5.75 ± 0.35</td>
<td>7.4</td>
<td>0.27 ± 0.03</td>
<td>1.37 ± 0.08</td>
<td>5.1</td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>1.21 ± 0.08</td>
<td>2.66 ± 0.37</td>
<td>2.2</td>
<td>0.89 ± 0.04</td>
<td>1.30 ± 0.03</td>
<td>1.5</td>
</tr>
<tr>
<td>Cytosol</td>
<td>7.28 ± 0.64</td>
<td>34.84 ± 4.23</td>
<td>4.8</td>
<td>0.46 ± 0.04</td>
<td>2.18 ± 0.26</td>
<td>4.7</td>
</tr>
</tbody>
</table>

in a rapid loss of PKC activity from the cytosolic fraction within 30 min (to ~20% of control). Concomitantly, there was a pronounced increase in plasma membrane-associated PKC activity (to ~370% of control). In contrast, the nuclear pool of PKC activity was not appreciably altered at early (30- and 90-min) time periods following exposure of the cells to TPA, although a rise (to ~160% of control) in nuclear PKC activity was noted at 3 h after the addition of TPA. Activity analysis also revealed that the time course and extent of down-regulation of PKC in the different subcellular fractions in response to prolonged exposure to TPA also are different. Cytosolic PKC was translocated and diminished within 30 min of exposure to TPA, although a residual (~20% of control) level of cytosolic PKC activity remained throughout the 24-h period of TPA treatment. The dramatic increase in plasma membrane-associated PKC was transient, and within 90 min after TPA treatment the PKC activity in the plasma membrane fraction had fallen by 60%. The plasma membrane PKC activity continued to show a drop through the remainder of the 24-h treatment period, but with only a maximal decrease to 40% of control. The nuclear pool of PKC, however, was completely down-regulated within 12 h of TPA treatment, and no PKC activity could be detected in the nuclear fraction at either the 12- or 24-h time periods. The observed differences in the time course and extent of TPA-induced redistribution and down regulation of PKC associated with the cytosolic, plasma membrane, and nuclear subcellular...
fractions strongly suggest that the nuclear pool of PKC found in MCF-7/ADR cells is distinct from the cytosol and plasma membrane pools of PKC in these cells.

Extraction of PKC from Isolated Nuclei. To determine whether the high level of PKC activity present in the purified nuclear fraction isolated from MCF-7/ADR cells is extractable by high salt concentration or by DNase treatment, the purified nuclei were first washed with 0.5 M NaCl or treated with 0.4 mg/ml DNase II for 1 h. These suspensions were centrifuged at 1000 x g for 10 min to obtain the extracted nuclear pellets, and these nuclear pellets then were resuspended in buffer containing 1% CHAPS detergent to solubilize remaining nuclear PKC. The detergent-solubilized nuclear fractions were assayed for PKC activity as described in "Materials and Methods" using PKC substrate peptide [(Ser<sup>19</sup>PKC<sup>31</sup>)] (GIBCO) as phosphoacceptor substrate. The detergent-solubilized PKC activities found in the nuclei pretreated as indicated are given as nmol <sup>32</sup>P incorporated/min/mg protein. Data are expressed as mean ± SEM.

Table 2  Detergent-solubilized PKC activity remaining in nuclei isolated from MCF-7/WT and MCF-7/ADR cells following either high salt concentration washing or DNase II digestion of the nuclear preparations

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Pretreatment with EB&lt;sup&gt;a&lt;/sup&gt; alone</th>
<th>Pretreatment with EB containing 0.5 M NaCl</th>
<th>Pretreatment with EB containing DNase II</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7/WT</td>
<td>0.171 ± 0.013</td>
<td>0.106 ± 0.023</td>
<td>0.100 ± 0.009</td>
</tr>
<tr>
<td>MCF-7/ADR</td>
<td>1.728 ± 0.046</td>
<td>1.175 ± 0.064</td>
<td>1.244 ± 0.059</td>
</tr>
</tbody>
</table>

* EB, equilibration buffer [20 mM Tris-Cl (pH 7.5), 2 mM EDTA, 2 mM EGTA, 2 mM dithiothreitol, and protease inhibitors (100 μg/ml leupeptin, 50 μg/ml aprotinin, 50 μg/ml (4-aminophenyl)methanesulfonyl fluoride, 10 μg/ml soybean trypsin inhibitor, and 100 ng/ml pepstatin A)].

Similar results were obtained with membranes from control MCF-7/WT cells and multidrug-resistant MCF-7/ADR<sup>im</sup> cells. These results indicate that the majority of PKC remains in the insoluble nuclear fraction and can be removed by detergent.

Correlation between Nuclear PKC Activity and Drug Resistance. The profound increase in nuclear PKC activity observed in multidrug-resistant MCF-7/ADR cells led us to investigate whether the level of nuclear PKC activity might correlate with the degree of drug resistance in MCF-7 cells. For this study, we used three different MCF-7 cell lines with different levels of drug resistance: one control MCF-7/WT cell line; and two drug-resistant MCF-7/ADR<sup>im</sup> and MCF-7/ADR<sup>cl</sup> cell lines designated as MCF-7/ADR<sup>im</sup> and MCF-7/ADR<sup>cl</sup> cells. The relative drug resistance of the three MCF-7 cell lines was determined by comparing the chemosensitivity of these cells to the cytotoxic effect of doxorubicin (Adriamycin) as determined by the MTT colorimetric assay as described in "Materials and Methods." Under the given culture and assay conditions, MCF-7/ADR<sup>im</sup> cells were the most resistant with an IC<sub>50</sub> of approximately 3 μM, while MCF-7/ADR<sup>cl</sup> cells exhibited a resistance (IC<sub>50</sub> ~ 0.3 μM) intermediate between MCF-7/ADR<sup>im</sup> cells and MCF-7/WT cells (Fig. 4; Table 3). Control, drug-sensitive MCF-7/WT cells exhibited an IC<sub>50</sub> of 0.04 μM Adriamycin.

The nuclear PKC activity of these three cell lines was determined, and the relationship between the nuclear PKC activity and the relative drug resistance was examined (Table 3). The subsequent detergent-solubilized nuclear PKC activity in both drug-sensitive MCF-7/WT cells and multidrug-resistant MCF-7/ADR cells.

![Graph showing Protein Kinase C Activity](image)

Fig. 3. Effect of TPA treatment of MCF-7/ADR cells on the distribution and down-regulation of protein kinase C activity found in the cytosolic, plasma membrane, and nuclear subcellular fractions. MCF-7/ADR cells were treated with 1 μM TPA for the indicated periods of time, the cells were harvested by scraping, and cytosolic, plasma membrane, and nuclear fractions were prepared as described in "Materials and Methods." Assays for PKC activity present in the subcellular fractions were carried out as described in "Materials and Methods" using PKC substrate peptide [(Ser<sup>19</sup>PKC<sup>31</sup>)] (GIBCO) as phosphoacceptor peptide. The detergent-solubilized PKC activities found in the nuclei pretreated as indicated are given as nmol <sup>32</sup>P incorporated/min/mg protein. Data are expressed as mean ± SEM.

![Graph showing Relative Sensitivity](image)

Fig. 4. Relative sensitivity of control MCF-7/WT cells and multidrug-resistant MCF-7/ADR<sup>im</sup> and MCF-7/ADR<sup>cl</sup> cells to the effect of 48-h treatment with increasing concentrations of doxorubicin (Adriamycin). Chemosensitivity of drug-sensitive MCF-7/WT cells and multidrug-resistant MCF-7/ADR<sup>im</sup> and MCF-7/ADR<sup>cl</sup> cells to the cytotoxic effect of doxorubicin (Adriamycin) was determined by MTT colorimetric assay as described in "Materials and Methods." Cells were treated with the indicated concentrations of doxorubicin (Adriamycin) for 48 h.
Most resistant cell line (MCF-7/ADR<sup>pm</sup>) had the highest nuclear PKC activity among these three cell lines, while the less drug-resistant MCF-7/ADR<sup>cf</sup> cell line showed an intermediate nuclear PKC activity. The control, drug-sensitive MCF-7/WT cells had the lowest nuclear PKC activity. It was of interest to determine the PKC activity among these three cell lines, while the less drug-resistant MCF-7/ADR<sup>cf</sup> cell line showed an intermediate nuclear PKC activity, and cytosolic and plasma membrane PKC activity and relative drug resistance in MCF-7 cell lines, we determined the PKC activities of various subcellular fractions prepared from a second set of independently isolated drug-sensitive and drug-resistant cells derived from additional MCF-7 cell lines [MCF-7(GY) cell lines, kindly provided by Dr. Grace Yeh]. Again, a good correlation was noted between the level of nuclear PKC activity and the relative degree of drug resistance exhibited by these MCF-7(GY) cell lines (Table 3). Although PKC activities associated with the plasma membrane and cytosolic fractions were also somewhat elevated in these drug-resistant cell lines (MCF-7/ADR<sup>cf</sup> and MCF-7/ADR<sup>pm</sup>) compared to that of the drug-sensitive MCF-7/WT cells.

To further establish the relationship between nuclear PKC activity and relative drug resistance in MCF-7 cell lines, we determined the PKC activities of various subcellular fractions prepared from a second set of independently isolated drug-sensitive and drug-resistant cells derived from additional MCF-7 cell lines [MCF-7(GY) cell lines, kindly provided by Dr. Grace Yeh]. Again, a good correlation was noted between the level of nuclear PKC activity and the relative degree of drug resistance exhibited by these MCF-7(GY) cell lines (Table 3). Although PKC activities associated with the plasma membrane and cytosolic fractions were also somewhat elevated in these drug-resistant cell lines, a close relationship between changes in cytosolic and plasma membrane PKC activity and relative drug resistance was not evident.

### Table 3 Relationship between nuclear protein kinase C activity and sensitivity to the cytotoxic effect of Adriamycin in drug-sensitive MCF-7/WT and multidrug-resistant MCF-7/ADR cells

<table>
<thead>
<tr>
<th>Cell type&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Protein kinase C activity&lt;sup&gt;b&lt;/sup&gt; (nmol &lt;sup&gt;32&lt;/sup&gt;P incorporated/ min/mg protein)</th>
<th>Sensitivity to Adriamycin (IC&lt;sub&gt;50&lt;/sub&gt; (µM))</th>
<th>Relative resistance to Adriamycin&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7/WT</td>
<td>0.25 ± 0.04</td>
<td>1.07 ± 0.08</td>
<td>1.07 ± 0.10</td>
</tr>
<tr>
<td>MCF-7/ADR&lt;sup&gt;cf&lt;/sup&gt;</td>
<td>1.02 ± 0.21</td>
<td>1.07 ± 0.10</td>
<td>1.07 ± 0.10</td>
</tr>
<tr>
<td>MCF-7/ADR&lt;sup&gt;pm&lt;/sup&gt;</td>
<td>1.12 ± 0.07</td>
<td>1.37 ± 0.08</td>
<td>1.37 ± 0.08</td>
</tr>
<tr>
<td>MCF-7/ADR&lt;sup&gt;cf&lt;/sup&gt;</td>
<td>1.70 ± 0.09</td>
<td>1.70 ± 0.09</td>
<td>1.70 ± 0.09</td>
</tr>
<tr>
<td>MCF-7/ADR&lt;sup&gt;pm&lt;/sup&gt;</td>
<td>2.70 ± 0.09</td>
<td>1.70 ± 0.09</td>
<td>1.70 ± 0.09</td>
</tr>
<tr>
<td>MCF-7/ADR&lt;sup&gt;cf&lt;/sup&gt;</td>
<td>2.68 ± 0.12</td>
<td>3.15 ± 0.08</td>
<td>3.15 ± 0.08</td>
</tr>
<tr>
<td>MCF-7/ADR&lt;sup&gt;pm&lt;/sup&gt;</td>
<td>2.68 ± 0.12</td>
<td>3.15 ± 0.08</td>
<td>3.15 ± 0.08</td>
</tr>
</tbody>
</table>

<sup>a</sup> For PKC activity measurements, histone H1 (type III-S) was used as substrate for MCF-7 cells received from Dr. Kenneth Cowan, and PKC substrate peptide (Gibco) was used as substrate for the (GY) series of cells.

<sup>b</sup> Data provided by Dr. Grace Yeh.

<sup>c</sup>Unless otherwise stated, all data were provided by Dr. Grace Yeh.

The elevated nuclear PKC activity found in MCF-7/ADR cells, we have compared the DEAE-cellulose chromatography elution profiles of the detergent-solubilized PKC activities obtained from nuclei isolated from MCF-7/WT and MCF-7/ADR cells (Fig. 5). The elution profile of nuclear PKC activity from MCF-7/ADR cells was quite different not only quantitatively, but also qualitatively, from that of MCF-7/WT cells. In MCF-7/WT cells, the nuclear PKC activity was very low and was mainly eluted from the column with 140 mM NaCl. Since the level of PKC activity present in the nuclear extract from MCF-7/WT cells was very low, this observation was confirmed from a similar elution profile obtained by applying three times the amount of nuclear protein on the column (Fig. 5, O). In contrast, MCF-7/ADR cells contained high levels of nuclear PKC activity which eluted in two major peaks from the DEAE column. The first peak eluted with 140 mM NaCl, while the second, more prominent, peak of activity eluted with 180 mM NaCl. Both peaks of nuclear PKC activity noted with the MCF-7/ADR extract were found to be immunoreactive with both the pan-specific PKC antiserum and with PKCα-specific antiserum by dot blot analysis (data not shown).

Western Blot Analysis of PKC in MCF-7/WT and MCF-7/ADR cells. Our finding that the increase in immunohistochemical staining and in PKC activity noted in MCF-7/ADR cells is due to an increase in PKC enzyme was further established by Western blot analysis of total cell extracts using a PKC antibody pan-specific for PKC isotypes α, β, and γ (Fig. 6). Markedly elevated levels of PKC protein were observed in MCF-7/ADR cells. We also examined the PKC isotype pattern present in the total cell extracts prepared from these cell lines with α, β, and γ isotype-specific PKC antibodies (Fig. 6). The antibodies used were raised in rabbits against homocyanin-conjugated synthetic peptides as described in “Materials and Methods.” α-isotype PKC (PKCa) and β-isotype PKC (PKCb) were found to be present in the MCF-7 cell lines. MCF-7/ADR cells contained markedly increased amounts of PKCa compared to that of MCF-7/WT cells. No significant difference in the amounts of PKCb and PKCγ was found in MCF-7/WT cells and MCF-7/ADR cells, and we were unable to detect PKCy in these cell lines. Recently, additional members of the PKC family (i.e., δ, ε, and ζ subtypes) have been identified (21, 22). Preliminary evidence with antisera specific to the δ, ε, and ζ isotypes of PKC indicates that none of these isoforms is elevated in MCF-7/ADR cells. Because of the different antibody preparations used, it is not possible to make a direct correlation between the amount of specific PKC isoforms and the level of PKC activity.
make a comparison between the amount of PKC\(\alpha\) relative to PKC\(\beta\) that might be present in the MCF-7 cell lines based upon the intensity of the bands. Results suggest that the pan-specific PKC antisera used recognize the PKC (apparently PKC\(\alpha\)) present in the MCF-7/ADR cells to a much greater degree than do the \(\alpha\)-specific PKC antisera.

Similar immunoblot patterns were observed with purified nuclei isolated from MCF-7/WT and MCF-7/ADR cells (Fig. 7). The purified nuclei isolated from MCF-7/ADR cells again revealed a marked increase in immunostaining with pan-specific PKC antibody and also contained elevated amounts of PKC\(\alpha\). As in the immunoblot analysis of total cell extract, there was little change in PKC\(\beta\), and we could not detect PKC\(\gamma\) in the nuclei prepared from these cell lines (data not shown).

When the CHAPS-solubilized nuclear protein fractions were electrophoresed on 10–20% SDS-polyacrylamide gradient gels, electrophoretically transferred to Immobilon-P membranes, and analyzed by immunoblotting with the PKC antiserum pan-specific for PKC isotypes \(\alpha\), \(\beta\), and \(\gamma\), the MCF-7/ADR nuclear extract revealed not only a marked increase in immunoreactivity but also a distinct difference in the immunostaining pattern (3 bands) when compared to that of MCF-7/WT cells (2 bands) (Fig. 8). The upper, major PKC band present in nuclei isolated from MCF-7/ADR cells was not detectable in nuclei isolated from MCF-7/WT cells. None of these bands was detected when immunoblots were performed with preimmune serum (data not shown). The reason for the difference in the pattern of PKC bands detected by Western blot analysis of solubilized nuclear proteins electrophoresed on 8% polyacrylamide gels (Fig. 7) compared to the immunostaining pattern noted with 10–20% polyacrylamide gradient gels (Fig. 8) is not readily apparent. Nonetheless, this result does further suggest that MCF-7/ADR cells do contain elevated levels of an apparently modified form of nuclear PKC.
protein were electrophoresed using 10-20% SDS-polyacrylamide gradient gels. Equivalent amounts (2 ng) of SDS denatured nuclear 7/W and MCF-7/ADR cells, and the solubilized protein fractions were dena-

bilibilized protein fractions were prepared from purified nuclei isolated from MCF-7/ADR cells and electrophoresed using 10-20% SDS-polyacrylamide gradient gels. Detergent (1% CHAPS)-solu-

nuclei isolated from MCF-7/WT and MCF-7/ADR cells and electrophoresed with rabbit anti-peptide antiserum pan-specific for a, ß, and y isotypes of PKC. and the protein bands then were electrophoretically transferred to Immobilon-P membranes. PKC immunoreactive proteins were detected by probing the blot.

PKC immunoreactive proteins were detected by probing the blot with rabbit anti-peptide antiserum pan-specific for a, ß, and y isotypes of PKC.

**DISCUSSION**

Several lines of evidence indicate the possible involvement of PKC in modulating cellular resistance to antitumor drugs of the natural products class. Protein kinase C activity has been reported to be elevated in several cell types exhibiting the multidrug-resistance phenotype, including MCF-7 human breast carcinoma cells (14), HL-60 leukemia cells (23, 24), murine fibrosarcoma cells (15), and KB human carcinoma cells (25). Results of cell growth and cytoxicity studies in which PKC activity has been altered with TPA and with the H-7 [1-(5-isooquinolinesulfonyl)-2-methylpiperazine] inhibitor also indicate a close relationship between PKC activity and cell sensitivity to the cytoxic effects of these anticancer drugs (14–17). Furthermore, studies suggest that PKC-activation may be involved in the phosphorylation of the membrane P-glycoprotein involved in drug transport. Enhanced phosphorylation of P-glycoprotein has been noted with phorbol ester treatment of K562/ADM cells (26), and Chambers et al. (25) have reported that the P-glycoprotein from drug-resistant human KB carcinoma cells can serve as a good substrate for PKC in vitro.

In this study, we have presented a further characterization of the enhanced levels of PKC found in multidrug-resistant MCF-7 human breast carcinoma cells when compared to that of control drug-sensitive MCF-7/WT cells. Our results indicate that MCF-7/ADR drug-resistant cells contain markedly elevated levels of nuclear PKC. This conclusion is based upon the following data: (a) immunohistochemical localization studies using a PKC antibody pan-specific for a, ß, and y isotypes of PKC indicated that immunoreactivity was enhanced in MCF-7/ADR cells compared to that of MCF-7/WT cells, with pronounced staining noted in the nuclear region; (b) other immunohistochemical studies with nuclei isolated from MCF-7 cells also revealed a significant increase in the intensity of immunostaining for PKC in nuclei isolated from MCF-7/WT cells; (c) MCF-7/ADR cells contained a markedly elevated level of nuclear PKC activity compared to that of MCF-7/WT cells; (d) another set of multidrug-resistant MCF-7 cell lines [MCF-

7(GY)/W0.3 and MCF-7(GY)/W10 cells] also was found to exhibit an increased level of nuclear PKC activity compared to that of control drug-sensitive MCF-7(GY)/WT cells; (e) Western blot analysis of total cell extracts with a pan-specific PKC antibody recognizing the a, ß, and y isotypes of PKC, as well as with a, ß, and y isotype-specific PKC antibodies, showed that MCF-7/ADR cells contained highly elevated amounts of PKCa when compared to that of MCF-7/WT cells, while no difference was observed in the amounts of PKCB and PKCy which was not detected in these cell lines; and (f) immunoblot analysis of proteins extracted from purified nuclei isolated from these MCF-7 cell lines revealed that nuclei isolated from MCF-7/ADR cells contained highly elevated levels of PKCa.

Protein kinase C has been found to be associated with the nuclear fraction of a number of cell types (18, 27–32). To establish that the markedly elevated level of PKC found in MCF-7/ADR cells represents a pool of PKC separate from the cytosolic and plasma membrane fractions, we carried out time course studies of changes in the distribution and down-regulation of PKC activity in the different subcellular fractions induced by treatment of these cells with 1 μM TPA (Fig. 3). When PKC activity had been down-regulated by prolonged (24-

h) exposure of MCF-7/ADR cells to TPA, significant PKC activity still remained in both the cytosolic (20% of control) and plasma membrane (40% of control) subcellular fractions. In contrast, there was a complete loss of the nuclear pool of PKC activity within 12 h after exposure of the cells to TPA. This, along with the differences noted in the rapid (30-min) redistribution of PKC from the cytosolic to the plasma membrane fraction, compared with no change detected in the nuclear PKC activity at 30 and 90 min of TPA treatment, strongly suggests that the nuclear pool of PKC present in MCF-7/ADR cells is distinct from the cytosolic and plasma membrane pools of PKC.

There appears to be a good correlation between the level of nuclear PKC activity found and the degree of drug resistance in MCF-7 human breast carcinoma cells. This was observed in two different MCF-7 cell lines: (a) MCF-7 cells obtained from Dr. Kenneth Cowan [control MCF-7/WT cells and multidrug-resistant MCF-7/ADR cells (MCF-7/ADR" and MCF-7/ ADR")]; (b) MCF-7 cells obtained from Dr. Grace Yeh [control MCF-7(GY)/WT cells and multidrug-resistant MCF-7(GY)/ W0.3 cells and MCF-7(GY)/W10 cells]. In both MCF-7 cell lines the most resistant cell types were found to contain the highest level of nuclear PKC activity, with less resistant cell types having intermediate levels of nuclear PKC activity relative to the very low level of nuclear PKC activity found in the control, drug-sensitive cell types. It should be noted that this quantitative correlation between the level of nuclear PKC and
ELEVATED NUCLEAR PKC AND DRUG RESISTANCE

the degree of drug resistance is masked when PKC activities determined in total cell homogenates are compared. A close relationship between the level of PKC activity present in the cytosol and plasma membrane and the degree of drug resistance was not evident.

Observations made during the course of this investigation indicate that the PKC activities found in the purified nuclear fractions isolated from MCF-7/WT and MCF-7/ADR cells exhibit somewhat different characteristics, suggesting that a different or modified form of PKC may be present in the nucleus of MCF-7/ADR cells. (a) The DEAE-cellulose column elution profiles of nuclear PKC isolated from MCF-7/WT and MCF-7/ADR cells revealed both quantitative and qualitative differences between these two activities (Fig. 5). With MCF-7/WT cells, the low amount of nuclear PKC activity detected was eluted with 140 mM NaCl. In contrast, two major peaks of nuclear PKC activity were observed with the extract from MCF-7/ADR cells. The first peak eluted at 140 mM NaCl, while the second, more prominent, peak of MCF-7/ADR nuclear PKC activity eluted at 180 mM NaCl. The explanation for the altered elution profile for the second, high salt peak of PKC activity noted with the nuclear protein fraction isolated from MCF-7/ADR cells remains to be established. Both changes in the phosphorylation state of PKC (33) and changes in the oxidative state of PKC (34, 35) have been reported to modify PKC to a form which elutes from DEAE-cellulose at higher (200–250 mM) salt concentrations. Modifications of this type may be responsible for the altered form of PKC found in nuclei isolated from MCF-7/ADR cells, and these possibilities are currently under investigation. (b) When the CHAPS-solubilized nuclear protein fractions of MCF-7/WT and MCF-7/ADR cells were electrophoresed using 10–20% polyacrylamide gradient gels, Western blot analysis revealed a distinct difference in both the intensity and pattern of PKC immunostaining between the two cell types (Fig. 8). The upper, highly stained PKC band found in the nuclear fraction of drug-sensitive MCF-7/WT cells is rapidly inactivated in response to treatment of these multidrug-resistant cells with H2O2 and with sangivamycin. Neither of these treatments results in a loss of PKC activity from the nuclear fraction of drug-sensitive MCF-7/WT cells. (c) Our preliminary data indicate that the nuclear pool of PKC present in MCF-7/ADR cells is rapidly inactivated in response to treatment of these multidrug-resistant cells with H2O2 and with sangivamycin.

In conclusion, the results presented indicate that drug-resistant MCF-7/ADR cells contain not only increased amounts of cytosolic PKC but also markedly elevated levels of an apparently modified form of PKC. The degree of drug resistance is masked when PKC activities determined in total cell homogenates are compared. A close relationship between the level of PKC activity present in the cytosol and plasma membrane and the degree of drug resistance was not evident.

ACKNOWLEDGMENTS

We are grateful to Dr. Kenneth Cowan (National Cancer Institute, NIH, Bethesda, MD) for generously providing us MCF-7/WT cells and MCF-7/ADR cells, and to Dr. Grace Yeh (National Cancer Institute) for kindly providing us MCF-7(GY)/WT cells, MCF-7(GY)/W0.3, and MCF-7(GY)/W10 cells. We also wish to thank Dr. Ulf Rapp (National Cancer Institute, Frederick County Research Facility, NIH, Frederick, MD) and Dr. Peter Blumberg (National Cancer Institute) for help and advice in the preparation and characterization of the PKC antisera used in this study.

REFERENCES


* Manuscripts in preparation.

Downloaded from cancerres.aacrjournals.org on April 14, 2017. © 1992 American Association for Cancer Research.
ELEVATED NUCLEAR PKC AND DRUG RESISTANCE


Elevated Level of Nuclear Protein Kinase C in Multidrug-resistant MCF-7 Human Breast Carcinoma Cells

Sung Ae Lee, James W. Karaszkiewicz and Wayne B. Anderson