Protein Kinase C in Adriamycin Action and Resistance in Mouse Sarcoma 180 Cells

James Posada, Paul Vichi, and Thomas R. Tritton

Department of Pharmacology and Vermont Regional Cancer Center, University of Vermont School of Medicine, Burlington, Vermont 05405

ABSTRACT

Adriamycin has a wide variety of biological actions on susceptible cells, several of which may be integrally involved in cytotoxicity. In this paper, we present evidence that one of the alterations in cell function that occurs in the presence of Adriamycin is an elevation in the production of diacylglycerol. The effect is rapid, reaches a peak within 10 min of exposure of Sarcoma 180 cells to Adriamycin, and can thus be classified among the earliest alterations that occur in cells damaged by Adriamycin. Concomitant with the rise in diacylglycerol is an increase in cytosolic protein kinase C activity. Although Adriamycin does not appear to modulate the activity of this enzyme by direct binding, drug-exposed Sarcoma 180 cells have a 56% increase in intrinsic cytosolic protein kinase C (PKC) activity, with no change in the activity of the membrane form. Experiments with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate suggest that the PKC effect is linked to Adriamycin action, since activation of the enzyme by short 12-O-tetradecanoylphorbol-13-acetate exposure enhances Adriamycin’s cytotoxicity as well as its ability to provoke DNA damage (measured by alkaline elution). Likewise, down-regulation of PKC by extended 12-O-tetradecanoylphorbol-13-acetate exposure partially protects the cells from Adriamycin-induced cytotoxicity as well as from DNA damage. Thus, the ability of cells to be injured by Adriamycin appears to be correlated with the activity of PKC. Multidrug-resistant subline Sarcoma 180A10 cells have the same total quantity of membrane-recruitable PKC as the sensitive parent Sarcoma 180 cells, as determined by [3H]phorbol-12,13-dibutyrate binding. However, the resistant cells have a significantly higher intrinsic PKC activity and an altered ability to translocate the enzyme to the cell surface. Taken together, the results raise the possibility that cell signaling mechanisms, particularly those involving protein kinase C, may play an important role in mediating the biological action of the anticancer drug Adriamycin.

INTRODUCTION

Providing a complete description of the mechanism of cytotoxic action of the antineoplastic drug Adriamycin has proven difficult because of the numerous biological effects elicited by this compound. For example, Adriamycin alters DNA structure by intercalation (1), stimulates the production of reactive oxygen (2), may itself be an alkylating agent (3), stabilizes the topoisomerase II-DNA cleavage complex (4), and damages plasma membrane function (5). Any of these events could conceivably lead to cytotoxicity, although they are not necessarily mutually exclusive. The possibility we begin to explore is that early action at the cell surface may precede and be linked to later events in the nucleus. We are particularly concerned with alteration of signal transduction mechanisms which regulate cellular proliferation when proceeding normally, but which may lead to cell injury or death when disrupted. The focus is on PKC because of the key role this enzyme plays in the transduction of mitogenic stimuli across the plasma membrane (6). We show that PKC activity in Sarcoma 180 cells is altered by Adriamycin and is associated with increased diacylglycerol production. Moreover, this modulation appears to be linked to the cytotoxic effect of the drug as well as the acquired ability of cells to resist the cytotoxic action.

MATERIALS AND METHODS

Cells. Sarcoma 180 cells were originally obtained from the American Type Culture Collection. S180A10 is a multidrug-resistant subline obtained by selection for resistance to Adriamycin (7). It is 125-fold resistant to the selecting agents. Both cell lines were grown in suspension with McCoy’s Medium 5A and 10% heat-inactivated horse serum in 5% CO2 incubators at 37°C; the S180A10 cells are grown in 0.89 μM ADR, which is removed several days prior to an experiment. All experiments were conducted on cells in early log phase (250,000 to 300,000 cells/ml) to minimize experimental variations which occur with proliferative status.

Diacylglycerol Assay. Cells in early log phase were washed once with medium, resuspended in fresh medium containing [3H]Arachidonic acid (1 μCi/ml) or [3H]oleic acid (1 μCi/ml), and incubated at 37°C for 6 h. Equal numbers of cells were then aliquoted into tubes containing either no addition or 5 μM Adriamycin and further incubated at 37°C for specified times. The lipids were extracted with CHCl3:CH3OH:EtOH (1:2:1) and dried under N2. The dried lipid film was resuspended in 60 μl of CH3OH, spotted into Whatman LK-5 silica gel thin-layer chromatography plates, and resolved using hexane:diethyl ether:acetic acid (50:50:1). Both the 1,2- and 1,3-diacylglycerol isomers were identified by comigration with known standards, and the 1,2-isomer was scraped from the plate and counted in Ecolumi scintillation cocktail. This procedure does not measure the absolute mass of diacylglycerol, but does reveal changes in the relative amount of this species.

Protein Kinase C Assay. The PKC assay of Kitano et al. (8) was used as follows. Cells were grown to early log phase as described above and then exposed to Adriamycin for varying times. Following treatment, the cells were washed in cold Buffer A [20 mM HEPES (pH 7.4): 2 mM MgCl2:2.5 mM EGTA:2 mM DTT:1 mM phenylmethylsulfonyl fluoride 50 μg/ml of leupeptin] and homogenized with 40 strokes in a Dounce homogenizer. Postnuclear supernatants were spun at 100,000 x g for 1 h; the cytosolic (soluble) PKC fraction was then applied to a 1-ml DE-52 column preequilibrated with Buffer B [20 mM HEPES (pH 7.4): 1 mM EGTA:1 mM DTT] and eluted with 0.1 M NaCl in Buffer B. The membrane (particulate) fraction was extracted with 1% Nonidot P-40, and spun at 100,000 x g, the supernatant was applied to a DE-52 column, and PKC was eluted as above. Five μg of cytosolic and membrane fractions were assayed for PKC activity in 26 mM HEPES, 10 mM MgCl2, 0.6 mM EGTA, 0.6 mM EDTA, 5.6 mM DTT, and 1 mg/ml of histone (Sigma; type III). Protein kinase C specific phosphorylation was determined by addition of 50 μg/ml of phosphatidylserine with adjustment of the free Ca2+ concentration to 0.5 mmol. The mixture was preincubated for 6 min at 30°C, and the reaction was initiated by addition of 50 μM ATP (250 to 300 cpm/pmol of ATP). Following a 3-min incubation, the reactants were immediately spotted on Whatman p81 phosphocellulose paper. The filters were then dipped into 75 mM phosphoric acid, washed 4 times, and counted in a Beckman liquid scintillation counter. Specific PKC activity is defined as the difference, in pmol of phosphate incorporated per μg of protein per 3 min, between the presence and absence of phosphatidylserine and calcium.

Cell Survival. The survival of S180 cells following exposure to...
Adriamycin was determined by cloning in soft agar as described by Chu and Fisher (9). Briefly, the cells were exposed to 0.1 to 5 μM ADR for 1 h, washed, and diluted to 50 cells/ml. Six-ml aliquots were placed in 60-mm Corning tissue culture dishes in 0.5% Difco Bacto agar. The plates were incubated 10 to 14 days at 37°C in a CO₂ incubator. The clones were counted using a low magnification dissecting microscope, and the raw counts were converted into cloning efficiencies using a computer spreadsheet. In this paper, survival due to a specified treatment is expressed as the percentage of increase or decrease relative to the appropriate control.

To determine the effects of down regulation of PKC on Adriamycin resistance, S180A10 cells were grown in 300 nM TPA for 36 h followed by exposure to specified doses of Adriamycin for 48 h. Cell growth inhibition by the drug is calculated relative to untreated control, or TPA-treated control for the phorbol ester down-regulated condition.

Alkaline Elution. DNA damage was assessed by quantifying DNA-protein cross-links as previously described (10). Growing cells were divided to 5 × 10⁶/ml in medium containing 0.5 μCi/ml of [³H]thymidine and 0.2 μCi/ml of [¹⁴C]thymidine to label DNA and grown for 14 h in a CO₂ incubator at 37°C. The cells were then washed and placed in fresh medium without isotope for an additional 12 h prior to an experiment. Preexposure to TPA (200 nM) was followed by washing and exposure to Adriamycin (0.1 to 5 μM) for various times. The cells were then lysed in lauryl sarcosine on polyvinylchloride filters and eluted under alkaline conditions. DNA-protein cross-links were calculated by comparison to a 3000-rad X-ray standard and expressed as rad equivalents of DNA damage.

Phorbol Binding. To measure phorbol ester binding to whole cells, we used an established procedure (11). The S180 or S180A10 cells were grown to early log phase and washed twice with McCoy's Medium 5A containing 1 mg/ml of bovine serum albumin. Tubes containing aliquoted amounts of [³H]PDBU were incubated with 3 × 10⁶ cells in 0.1 ml at 37°C for 15 min (time course studies showed that equilibrium was reached in 5 min). The cells were spun in a Beckman microfuge to separate free from bound phorbol and washed, and the pellet was counted in a liquid scintillation counter. Nonspecific binding was experimentally estimated by addition of a 1000-fold excess of cold PDBU. The data were analyzed using a version of the LIGAND program described by Munson and Rodbard (12).

RESULTS

Stimulation of Diacylglycerol Production. One of the key regulatory events in the control of cellular proliferation involves phospholipid, particularly phosphoinositide, turnover (13). The hypothesis we tested is that alteration of cell-signalling pathways by Adriamycin could lead to a loss of the ability of a cell to correctly control its growth, i.e., drug-induced cytotoxicity. Phosphoinositide turnover results in the production of the second messenger DG. Fig. 1 shows that the relative amount of diacylglycerol production increases following treatment of S180 cells with 5 μM Adriamycin. This effect is relatively rapid, reaching a peak in 10 min and beginning to decline by 60 min following drug exposure.

Since DG may arise from the action of a phosphatidylcholine-specific phospholipase C (14) as well as phosphoinositide-specific phospholipase C, labeling was done with [³H]arachidonic acid or [¹⁴C]oleic acid. The oleic acid will not be an appreciable fraction of the acyl side chains of the inositol lipids, while the arachidonic acid will be (15). The characteristics of the diacylglycerol response following Adriamycin treatment were independent of the fatty acid label used. This result suggests that the Adriamycin-induced diacylglycerol originated from a least two types of phospholipid, since activation of only one of either phosphatidylcholine- or phosphoinositide-specific phospholipase C would be expected to yield qualitatively different results depending on the fatty acid label used.
activity of the cytosolic form of PKC, with no change in the activity of the membrane PKC, and no shift in the subcellular enzyme distribution. This is not an unprecedented biological response, since both concanavalin A and ACTH treatment have been shown to induce similar results (16, 17).

Effect of Protein Kinase C Activity on Adriamycin Action. Since Adriamycin alters PKC activity in S180 cells we wondered whether the inverse would also be true, i.e., does modulation of PKC activity alter the cytotoxic ability of Adriamycin? To test this possibility we used the phorbol ester TPA to alter PKC activity. Figure 3A shows results of Adriamycin-induced cytotoxicity experiments where TPA pretreatment of the cells causes either reduction or enhancement of PKC activity. For example, 30-min 200 nm TPA pretreatment, which is without effect on cell growth but which increases the activity of membrane-bound PKC by translocation (see Fig. 4), followed by Adriamycin for 1 h, enhances the cytotoxic action of the drug at all doses tested. Conversely, down regulation of PKC activity with a 24-h pretreatment of TPA, which results in a 97% loss in enzyme activity (data not shown), decreased the cytotoxic effect of Adriamycin at all doses. Furthermore, when PKC activity and Adriamycin cytotoxicity are enhanced by TPA, alkaline elution-detected DNA-protein cross-links are also enhanced (Fig. 3B). Likewise, down regulation of PKC and protection from Adriamycin-induced cytotoxicity reduces the level of DNA damage. TPA itself has no effect on DNA-protein cross-links under the conditions of these experiments. Taken together, these results suggest that the activity of PKC appears to be coupled to the ability of cells to sustain DNA damage and be injured by Adriamycin.

PKC Activity in Multidrug-resistant S180 Cells. If PKC is implicated in the cytotoxic action of Adriamycin, it may also play a role in regulating resistance to Adriamycin. Fig. 4 compares the basal PKC activity in sensitive S180 cells to a 125-fold Adriamycin- and multidrug-resistant subline, S180A10. The results show that, while the membrane form of the enzyme has identical activity in both cell lines, the cytosolic activity is increased by 82% in the drug-resistant cells. In addition, the ability of TPA to cause translocation is attenuated in the MDR cells, since at equal doses of the phorbol ester a smaller fraction of the PKC activity is recruited to the cell membrane in the resistant cells.

**Fig. 4.** Basal protein kinase C activity in S180 and S180A10 cells. The cytosol and membrane PKC of each cell line was obtained as described in “Materials and Methods” and preincubated for 6 min at 30°C. TPA treatment was at 200 nmol for 30 min. The reaction was initiated by addition of [3H]ATP and terminated after 3 min by placing on ice. Bars SEM of replicates. Similar results were obtained in three independent experiments. The Ca²⁺ and phosphatidylserine independent kinase activity (in the same units as above) was: A, S180, cytosol = 6.5 ± 0.4, membrane = 7.9 ± 0.4; B, A10, cytosol = 7.8 ± 1.7, membrane = 7.0 ± 0.2. It is evident that there is a net loss in the measured total PKC activity following TPA treatment. Other investigators also observe this result (e.g., 40, 41), which probably represents TPA-induced aggregation of PKC with the detergent-insoluble fraction, like the cytoskeleton.*

The idea we sought to test in this work was that cellular signal transduction mechanisms might be disrupted by a cytotoxic agent, and such disruption could be coupled to cell death. Cell growth is regulated by a dynamic interplay involving numerous positive and negative signalling pathways. Among the regulatory elements are receptors for growth factors, mitogens, and hormones as well as associated signal transduction apparatuses such as protein phosphorylation, lipid methylation, cyclic nucleotide generation, ion fluxes, and turnover of phosphoinositides. Regulated, coordinated cell growth requires a delicate balance between these types of signals and events, and its plausible that disruption of such a finely regulated system

* S. Jaken, personal communication.

**Fig. 3.** A. TPA effect on Adriamycin cytotoxicity. S180 cells pretreated with 200 nm TPA for 30 min, to activate PKC, or 24 h, to down regulate PKC, were subsequently treated with the indicated concentrations of ADR for 1 h. Cell survival was determined by cloning in soft agar, and the results are presented as a percentage of change compared with control cells which received only Adriamycin treatment. B. TPA effect on Adriamycin-induced DNA damage. The cells were treated with 200 nm TPA followed by 1 μM Adriamycin as in A, and then DNA-protein cross-links were measured by alkaline elution.
could have disastrous consequences to a cell, *i.e.*, cytotoxicity. Our results indicate that an early response of cells exposed to the cytotoxic anticancer drug Adriamycin is a rapid rise in intracellular diacylglycerol. It is probable that the Adriamycin-induced diacylglycerol originates from at least one of two types of phospholipid, since labeling protocols involving [3H]arachidonic acid, which is one major constituent of the acyl side chains of inositol lipids, display the same qualitative result as labeling with [3H]oleic acid, which is distributed among many non-inositol-containing phospholipids, especially phosphatidylcholine (15). We have not quantified the fractional contribution of each source to the diglyceride pool because the point we wish to stress is that this messenger molecule appears in response to short ADR exposures, and regardless of the source, DG is available to interact with protein kinase C. The mechanism whereby Adriamycin causes the increase in diacylglycerol is not known. The idea we favor is that the DG production is stimulated by the drug's well-known ability to modulate membrane structure and fluidity (5), but the formation of oxygen-based free radicals is also a possible explanation (2).

The major identified biological action of the second messenger diacylglycerol is binding to PKC such that the Ca²⁺ requirement for activation is decreased (18, 19). In contrast to the action of the phorbol esters in stabilizing membrane association of PKC (*i.e.*, translocation), many agents which result in diacylglycerol production do not recruit PKC to the particulate fraction; indeed cell permeant diacylglycerol analogues themselves induce only a very small increase in membrane PKC activity (20). Viewed in this light, it is not surprising that Adriamycin treatment does not result in measurable translocation. The increase in cytosolic PKC rather than membrane PKC, while unusual, has also been reported for at least two other agents: concanavalin A (16) and adrenocorticotropic hormone (17).

It is unlikely that the rise in PKC activity is due to direct binding of the drug to the enzyme, since there is no Adriamycin effect on the enzyme activity *in vitro*. It is also doubtful that diacylglycerol itself is the sole mediator of Adriamycin's action since exogenously added DG is not cytotoxic. On the contrary, DG release is often associated with a proliferative response to growth factors and other mitogens (13). Therefore the observed Adriamycin effect appears to be anomalous with respect to PKC activation, and this disruption of normal cellular function may play a role in drug-induced cytotoxicity.

One possible explanation for the results is that Adriamycin interferes with the proteolysis of PKC resulting in a net increase in kinase activity. Another possibility arises from the fact that phospholipase A₂ activation is among the numerous membrane actions of Adriamycin. Phospholipid hydrolysis by this enzyme yields fatty acids, lysophospholipids and, eventually, prostaglandins (21, 22). Interestingly, fatty acids (23) and lysophosphatidylcholine (24) are activators of PKC, and the latter species acts in synergy with diacylglycerol in activating PKC (24). We reasoned that since Adriamycin affects the activity of PKC, then the intrinsic activity of the enzyme might control the cytotoxic action of the drug, if cytotoxicity and PKC activity are functionally coupled. The results suggest that this is the case since activation of PKC with phorbol ester treatment also increases the cytotoxic activity of Adriamycin. Likewise, down regulation of the PKC activity decreases the responsiveness of the cells to drug treatment. Previous work by Ferguson and Cheng (25) also showed protection from cytotoxicity by phorbol ester treatment. Another consideration in this line of thinking is our finding that modulation of PKC and Adriamycin's biological activity simultaneously alters the production of DNA damage; increased cytotoxicity is associated with more DNA-protein cross-links and decreased cytotoxicity with fewer DNA lesions. We consider this set of finding intriguing circumstantial evidence for the possibility that modulation of protein kinase C activity by Adriamycin leads to an alteration of lethal DNA damage. Consistent with this interpretation is the report (26) that topoisomerase II, an enzyme implicated in producing DNA-protein cross-links (27), is a putative substrate for PKC. Thus, early events at the cell surface (production of diacylglycerol) may precede and be linked to subsequent damage in the nucleus (via topoisomerase) which results in cell death.

If protein kinase C plays a role in mediating the cytotoxic response to Adriamycin, this same enzyme could also be a

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**Fig. 5.** [3H]PDBu binding to S180 and S180A10 cells. A, self-displacement binding curve. B, Scatchard analysis of data in A. S180 (○); S180A10 (●). The mean values of four independent experiments were: $K_a$, 9.2 ± 2.8 nM; 8.3 ± 4.8 nM; capacity, 0.13 ± 0.05 pmol/10⁶ cells, 0.10 ± 0.06 pmol/10⁶ cells for S180 and S180A10, respectively. Statistical analysis was performed by LIGAND (13). For the four data sets $F = 0.83$, $P > 0.5$.

**Fig. 6.** Adriamycin dose-response curve for control and TPA-treated drug-resistant cells. Multidrug-resistant S180A10 cells were cultured with 300 nM TPA for 36 h to cause down regulation of PKC. This was followed by exposure to Adriamycin for 48 h at the specified doses and counting of the cells with a Coulter Counter. Bars, standard deviation.
factor in the ability of a cell to resist cytotoxicity. The S180A10 subline was selected for resistance to Adriamycin and also exhibits the multidrug-resistant phenotype (7). The subline is 125-fold resistant to Adriamycin, shows cross-resistance to other structurally unrelated agents like vincristine and actinomycin D, and expresses elevated levels of P-glycoprotein in its plasma membrane. The resistant line also has elevated basal cytosolic PKC activity as compared with the sensitive lines, with identical membrane PKC activities. Using an MCF-7 multidrug-resistant cell line, Fine et al. (28, 29) also showed elevated total PKC activity in the resistant cells, but the increase was represented equally in both soluble and particulate fractions; i.e., the ratio between the two forms was not altered in the drug-resistant cells.

In this study we have established that an early effect of Adriamycin is a rapid and transient flux of diacylglycerol and elevated cytosolic PKC activity. Furthermore, we also find an increase in total PKC activity in the MDR cells which is similarly associated with the soluble PKC fraction. This increase in cytosolic PKC activity is not associated with an increase in the number of molecules of enzyme which can be recruited to translocate to the plasma membrane. Since multidrug-resistant S180A10 cells exhibit an increase in PKC activity similar to that seen following Adriamycin treatment, we hypothesize that a primary means of overcoming cytotoxic stress is by the stimulation of mitogenic machinery.

Associated with an increase in cytosolic PKC activity in the drug-resistant cells is an anomalous translocation response to phorbol ester. In drug-sensitive cells, the fraction of the total PKC which is soluble is 0.65, and this declines to 0.27 (change = 140%) when TPA is added to recruit the soluble form to the membrane. In the resistant cells, by contrast, this fraction declines from 0.76 to 0.52 (change = 46%). Thus it appears that the increase in basal PKC activity in the MDR cells is associated with decreased affinity for the plasma membrane. It can be seen in Fig. 4 that, although the fractional change is different, the same number of units of PKC are translocated in both cell lines by 200 nM phorbol ester. One might have expected that more PKC would be translocated in the MDR cell line, commensurate with the nearly two-fold increase seen in total PKC activity in these cells. However, this is not the case, since the same absolute amounts is translocated by both cell lines at 200 nM phorbol ester. To probe this result further, the full binding curve of Fig. 5 was done in order to translocate PKC over a wider dose range of 1 to 100 nM phorbol ester. The results clearly show that the same amount of PKC can be recruited to the membrane (i.e. translocated) at any given dose of phorbol ester in each cell line. Relative to the total PKC activity, this translocation response represents a smaller fraction of the enzyme activity in the MDR cell line as compared with the parental cell line. This suggests either that the additional PKC activity in the drug-resistant cells is functionally different from sensitive cells, or that the plasma membrane of the MDR cells may be altered in a way that affects PKC. In regard to this latter possibility, it is possible that the altered lipid composition associated with MDR (30) or the presence of large quantities of P-glycoprotein (31, 32) may affect the interaction of PKC with the membrane.

Since the MDR cells have increased cytosolic PKC, but similar membrane activity, we wanted to investigate the biological effect of increasing the amount of membrane bound activity on the MDR phenotype. This was done by treating drug-resistant cells for 36 h with TPA followed by exposure to Adriamycin. The result of the TPA pretreatment is to deplete cells of Ca2+ and phospholipid-stimulated kinase activity, most probably by membrane-associated proteolysis of PKC to its constitutively activated 50kD catalytic domain, M-kinase (33, 34). Under these circumstances the 50% lethal dose for Adriamycin increases approximately 3-fold. This result is of particular interest since the MDR cells have an abundance of a M, 170,000 protein (P-glycoprotein) thought to be an active efflux pump for cytotoxic drugs, and which may also be a substrate for PKC (35, 36). In addition, Aquino et al. have demonstrated a PKC-related M, 50,000 protein thought to be M-kinase following long-term TPA exposure, which appears to be linked to increased phosphorylation of the membrane protein vinculin (37).

In summary, we have shown that the diacylglycerol-protein kinase C signal transduction pathway may play a role in the cellular response to the cytotoxic agent Adriamycin. The situation is complicated by the fact that the enzyme exists in several isoforms with different cofactor and substrate requirements (38, 39). However, exploitation of these and related growth control processes represents a new approach toward designing specific anticancer drugs.

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