P-Glycoprotein, Multidrug Resistance and Protein Kinase C

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Key Words. Protein kinase C · P-glycoprotein · Multidrug resistance

ABSTRACT

The multidrug resistant (MDR) phenotype is a well-studied subject that has been recognized as a determinant underlying specific types of drug resistance in human cancer. Although it is clear that the P-glycoprotein plays a major role in MDR, it is not clear whether post-translational modifications such as phosphorylation have any major impact on its modulation.

The laboratory of Dr. Bruce Chabner was one of the first to describe increased expression and activity of protein kinase C (PKC) associated with the MDR phenotype. Since that time, a similar correlation has been observed in many other MDR cell lines. Most of these studies have been performed with doxorubicin-selected cells that have acquired MDR and have shown increased PKC activity, mainly for PKC-α isoenzyme. Intrinsic MDR in human renal cell carcinoma lines has been shown to correlate directly with PKC activity, but further studies with intrinsic MDR cell lines are needed before any conclusions can be drawn.

More recent evidence suggests that there is a complex biochemical process by which PKC isoenzymes differentially phosphorylate specific serine residues in the linker region of P-glycoprotein which may lead to alterations in P-glycoprotein ATPase and drug-binding functions. To further complicate matters, PKC plays an important role in anti-apoptotic pathways, which can confound the dissection and elucidation of drug-resistance mechanisms. However, these areas are still under active investigation and not fully answered. Further studies are needed to specifically answer the question of whether PKC directly modulates basal and/or drug-stimulated P-glycoprotein function.

This manuscript reviews the majority of the literature on PKC and MDR, as well as offers caveats for interpretation of these studies to answer the above questions. The Oncologist 1996;1:261-268

INTRODUCTION

The aim of this manuscript is to summarize some of the pertinent findings on the association and possible role of protein kinase C (PKC) upon P-glycoprotein function. Information will be drawn from multiple sources, including work performed in the laboratory of Dr. Bruce Chabner.

Tumor cells develop drug resistance through multiple mechanisms, and more than one mechanism of resistance is most likely operative in a single cancer cell at a particular point in time. One form of drug resistance, termed ‘multidrug resistance’ (MDR) leads to the simultaneous cross-resistance to several structurally unrelated natural products. Unfortunately, approximately 50% of antineoplastic agents within the armamentarium of the oncologist are derived from natural sources and fall under the umbrella of the MDR mechanism. The MDR phenotype confers resistance to the anthracyclines, vinca alkaloids, epipodophyllotoxins, taxanes, antibiotics and some of the new topo-I inhibitors. The mechanism(s) of resistance to these natural products is due to an energy-dependent drug efflux which results in a net decreased intracellular drug accumulation. Malignant mammalian cell lines with the MDR phenotype may have an amplified mdr1 gene (not common), which codes for a 4.2 kDa mRNA, or increased protein product of the mdr1 gene called P-glycoprotein (common). The P-glycoprotein is usually expressed as a 150 to 180 kDa transmembrane protein. There are two cytoplasmic ATP binding sites in the P-glycoprotein, which allow for hydrolysis of the gamma phosphate of ATP to provide the energy for drug efflux. The protein interdigitates the plasma membrane at 12 sites, and the
first extracellular transmembrane region is glycosylated [1-4]. Glycosylation is not thought to be an important factor in the mechanism by which this protein functions in the membrane, but may be important for proper routing to the cell surface.

An interesting feature of the MDR phenotype is its susceptibility to inhibition by certain nonnatural product agents. Calcium channel blockers, calmodulin antagonists, immunosuppressants, hormonal drugs and other agents have been found to inhibit P-glycoprotein function and the MDR phenotype. Structural similarities in many of these reversal agents include a ternary nitrogen, aromatic ring and amphipathic characteristics [5, 6]. The mechanism of action is thought to be direct binding to the P-glycoprotein and displacing natural product anticancer drugs, which increase intracellular drug accumulation. Some of the above agents also have other activities, which include inhibition of various protein kinases, namely, PKC.

Dr. Chabner’s laboratory had initially described increased PKC protein and activity in MDR human breast cancer cells, as well as induction of a transient MDR phenotype by phorbol ester activation of PKC in wild-type, control human breast cancer cells. This was associated with decreased intercellular drug accumulation and increased drug resistance in clonogenic assays [7]. Treatment of wild-type cells has also been shown to induce a transient MDR phenotype in other human carcinoma lines [8]. Subsequently, a number of laboratories reported increased activity and amount of PKC in MDR lines such as sarcoma-180 [9], murine fibrosarcoma [10], P-388 leukemia [11], HL-60 leukemia [12], human KB-carcinoma cells [13] and murine DCF-3F cells [14].

As will be discussed, protein kinase inhibitors which modulate MDR have also been noted to alter P-glycoprotein phosphorylation in association with inhibition of P-glycoprotein transport of substrates. Although other kinases have been shown to phosphorylate P-glycoprotein in vitro, there is considerable experimental evidence which suggests that phosphorylation of P-glycoprotein by PKC may play a prominent role in regulating MDR. In vitro, P-glycoprotein is phosphorylated by PKC at serine residues, primarily in the linker region of P-glycoprotein [15, 16].

The family of PKC isoenzymes has at least 11 members which can be classified into three groups: A) common PKC isoenzymes (alpha [α], beta-I [β-I], beta-II [β-II], gamma [γ]) which are calcium-dependent and phorbol ester-responsive; B) novel PKC isoenzymes (delta [δ], epsilon [ε], eta [η], theta [Θ] and mu [μ]) which are calcium-independent and phorbol ester-responsive, and C) atypical PKC isoenzymes (zeta [ζ], lambda [λ]) which are calcium-independent and phorbol ester-unresponsive [17].

Differences in expression, substrate specificity and activator requirements suggest that PKC isoenzymes may have distinct roles in different signaling pathways [18].

**INCREASED EXPRESSION OF PKC IN SELECTED MDR TUMOR CELL LINES**

As mentioned earlier, there are multiple reports of increased PKC activity present in drug-selected MDR cell lines in comparison to drug-sensitive parent cell lines [7-14]. In each of the cell lines studied, the level of PKC activity was found to be increased in the MDR drug-selected cell line relative to the parent cell line. These included *vinca* alkaloid-selected, as well as anthracycline-selected MDR cell lines. However, there is only a marginal increase in PKC activity found in P-388 leukemia cell lines that were drug-selected with anthracyclines or *vinca* alkaloids [14]. Also, in P-388 murine leukemia cell lines selected with VP-16 and in the MOLT leukemia line selected with trimetrexate, PKC activity level was actually lower than the level found in the parent cell line [19, 20]. Collectively, these reports suggest that, in the P-388 MDR cell line, the MDR phenotype is not associated with increased PKC activity, irrespective of the selecting drug selected, whether it is vincristine, doxorubicin, VP-16 or trimetrexate. However, the data do strongly suggest that doxorubicin and *vinca* alkaloid-selected tumor cells other than P-388 generally have elevated PKC activities associated with the MDR phenotype. PKC activity levels are not only elevated in the cytosolic compartment, but may be elevated in the nuclear compartment as well in at least one MCF-7 MDR cell line [21]. Murine sarcoma S180 cells exposed to doxorubicin for one hour were found to have markedly increased PKC activity and content, which suggested that PKC activation and elevation may be associated with early events in the selection of MDR tumor cells by exposure to doxorubicin [9]. This idea is corroborated by the observation that the level of PKC activity directly correlates with the MDR phenotype in a series of murine fibrosarcoma UV-2237M cell lines with different degrees of MDR [22, 23]. In these studies, the level of PKC activity directly correlated with the degree of MDR. However, a caveat may be offered here in that these are still only associations and are not direct proof that PKC directly modulates P-glycoprotein or affects the MDR phenotype. It can be said, at this point, that this is an interesting association which may also be an epiphenomenon because agents such as doxorubicin have more than one mechanism of action. It is conceivable that the free radical-generating ability of doxorubicin through one- and two-electron transfers may select for MDR cells which have altered forms of PKC that are less susceptible to catabolism. Cells which survive doxorubicin exposure in vitro may have PKC isoforms which are more resistant to free-radical damage and thus express decreased catabolism and result in a net increase in enzyme content. It is also possible that PKC may affect non-MDR mechanisms of resistance which have not been thoroughly investigated, such as the apoptotic pathway which is known to be inhibited by increased activities of PKC in certain cell types.
INHIBITORS AND ACTIVATORS OF PKC AND EFFECT UPON MDR

Treatment of MDR cells with PKC activators has been reported to increase phosphorylation of the P-glycoprotein [24-28] and decrease drug accumulation [7, 27, 29] and drug sensitivity [7, 29]. Conversely, treatment of MDR cells with PKC inhibitors has been shown to decrease phosphorylation of P-glycoprotein [24, 27, 30], drug-efflux activity [27, 31, 32] and P-glycoprotein drug binding [27, 30, 32]. However, some PKC inhibitors such as calphostin-C also bind to the P-glycoprotein [27]. Calphostin-C has been shown by the laboratory of Dr. Susan Bates to decrease PKC ζ in SW620 Ad300 cells [24]. Thus, in studies which used PKC inhibitors that also bind to P-glycoprotein (such as staurosporine and calphostin-C), the possibility exists that inhibition of P-glycoprotein function results either from inhibition of drug binding or from inhibition of P-glycoprotein phosphorylation by direct or indirect effects on PKC.

Recent work performed in our laboratory demonstrated that safinogol, a lysosphingolipid derivative which specifically inhibits PKC activity via competitive interaction with the regulatory phorbol-binding domain of PKC, can inhibit the MDR phenotype without altering P-glycoprotein drug binding [33]. Our research indicates that safinogol and its lysosphingolipid analogs do compete with the phorbol-binding sites on PKC, but do not compete for azidopine photo-affinity labeling sites, do not compete with vinca alkaloids for vinblastine-binding sites and do not interact or activate the P-glycoprotein ATPase function like vinblastine or verapamil. These recent findings are novel because they strongly suggest that there is a second mechanism for regulation of the P-glycoprotein activity, namely, phosphorylation of P-glycoprotein by PKC.

Work from the laboratory of Dr. Igor Roninson has shown induction of MDR1 mRNA and P-glycoprotein in certain wild-type mammalian cancer cells and normal lymphocytes after short-term exposure to various agents, including phorbol esters, anthracyclines and vinca alkaloids. This induction of MDR required moderate to high concentrations of the agent which nearly induced death in most cells. Interestingly, this induction of MDR could be blocked by PKC inhibitors which the authors suggested as evidence for a role of PKC in regulating expression of P-glycoprotein [34, 35]. PKC may be more directly involved in activation of the mdr1 gene since it has AP-1 sites in its promoter which may interact with PKC. This important and novel data could also be explained, by our interpretation, as part of the “toxin response” observed in cells exposed to lethal damage which induces a myriad of biochemical responses in the cell to survive, including MDR, bcl-2, glutathione, etc., which help block apoptosis. If these responses do not “save” the cell, then apoptosis ensues. PKC activation is known to be involved in anti-apoptotic pathways, and inhibitors of PKC promote apoptosis. Thus, it is possible that inhibitors of PKC may block or abrogate anti-apoptotic pathways such as MDR that then lead to cell death. This area of research has important implications and requires further investigation.

Further work on the regulation of the mdr1 promoter has shown that it can be activated by the addition of serum, platelet-derived growth factor and TGF-α to quiescent 3T3 NIH cells transfected with mdr1, which are stimulated into cell cycle. Raf kinase, or v-raf, transfection led to a tenfold increase in mdr1 promoter activity. These data suggest that the mdr1 gene may be transcriptionally regulated by cellular signaling events, including raf kinase [36].

PKC ISOENZYMES IN MDR

There are a number of studies which have demonstrated a specific isoform of PKC which is overexpressed in MDR-selected cell lines. Most of these studies have shown PKC-α to be the overexpressed isoform. As previously noted, the MDR UV-2237M, human carcinoma KB-A10, murine sarcoma S180 and human breast cancer MCF-7 cells have all been shown in their MDR sublines to have increased PKC with specific increases in PKC-α activity and immunoreactivity and, in some cases, increased PKC-α message. Alternatively, PKC activity which is elevated in the MDR HL-60/ADR leukemia subline, which contains about two to three times as much immunoreactive PKC as the parental HL-60 cell line, has no elevation in PKC-α in the MDR subline, but PKC-β is decreased and PKC-γ is present exclusively in the MDR-resistant line by Western analysis [12, 37].

Recent collaborative research with the laboratory of Dr. Yusef Hannun has demonstrated reciprocal changes in calcium-dependent and calcium-independent PKC activity in the doxorubicin-selected MDR MCF-7 cell line. Expression of PKC-δ and -ε isoenzymes (calcium-independent PKC activity) was shown to be decreased in MDR cells relative to drug-sensitive MCF-7 cells. In contrast, increased expression of mRNA and protein for PKC-α isoenzyme in MDR MCF-7 cells was associated with increased calcium-dependent PKC activity and enhanced phosphorylation of the myristolated alanine-rich C-kinase substrate (MARCKS), and P-glycoprotein [26].

Transfection of MDR1 transfected MCF-7 cells with PKC-α was shown to further increase resistance to natural-product drugs in association with reduced accumulation and enhanced phosphorylation of P-glycoprotein [38]. Further evidence indicating that PKC-α-isoenzyme is a positive regulator of P-glycoprotein function in breast cancer cells was suggested by the finding that expression of PKC α-antisense cDNA decreased PKC activity, P-glycoprotein phosphorylation and the MDR phenotype [39]. Comparisons

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of PKC isoenzyme expression in other paired-sensitive and drug-selected MDR cell lines have also shown increased expression of PKC-α [24, 28, 38, 39], PKC-β [11], and PKC-γ [39].

The mechanism by which PKC isoforms increase in MDR cell lines is not well understood. Though PKC-α is the most commonly overexpressed isof orm, it is not clearly established which isoform seems to be the most important in correlation or association with the MDR phenotype. This phenomenon appears to be cell-type specific. However, the mechanism of PKC-α overexpression in the doxorubicin-selected MDR UV-2237M murine fibrosarcoma cell line does appear to entail a reduced rate of PKC degradation [10]. Phorbol-induced downregulation of PKC by increased proteolysis of the enzyme does not occur to the same degree in this MDR cell line as in the wild-type cell line. This defective phorbol-mediated downregulation of PKC-α strongly suggests that a reduced proteolysis of PKC contributes to its overexpression in the MDR cell line and is not due to increased synthesis of the protein [10]. This is further corroborated by studies in MDR KB-V1 cells which also undergo incomplete downregulation of PKC when exposed to phorbol esters as compared to the parent cell line, KB-3 [40]. Both of these MDR cell lines were developed from exposure to vinca alkaloids, and it is possible that there is a decreased rate of PKC degradation in certain cell lines which are exposed to vinca alkaloids to produce MDR cell induction [40]. Another possibility is that PKC-α is differentially regulated by proteolysis in MDR cells [10, 40]. Whatever the mechanism, it is clear that in some cell lines, such as MDR-transfected MCF-7 and rat fibroblasts, transfected PKC-α overexpression produces further protection against cytotoxic drugs as effectively as phorbol ester exposure [41].

Coexpression of PKC-α and P-glycoprotein in a baculovirus expression system in insect Sf9 cells demonstrated that PKC-α and P-glycoprotein were tightly associated in membrane vesicles when they were co-immunoprecipitated with antibodies against either PKC-α or P-glycoprotein. The P-glycoprotein was also phosphorylated in the baculovirus coinfection in the insect Sf9 cells. Interestingly, azidopine photo-affinity labeling to P-glycoprotein and its ATPase activity was moderately increased with coinfection of PKC-α. Addition of the PKC inhibitor RO31-8220 inhibited the stimulatory effects of PKC-α upon the P-glycoprotein ATPase activity. Mutation of serine 671 to asparagine in the linker region of P-glycoprotein abrogated verapamil and PKC-α-stimulated ATPase activity. Basal ATPase activity (nondrug stimulated), was not affected by this mutation. The authors concluded that PKC-α exerts a positive regulation upon P-glycoprotein ATPase activity and to its binding of substrate [42].

Another area that has not been well explored is the role of M-kinase in MDR. The M-kinase is the proteolytic, catalytic subunit of PKC that lacks phorbol-binding sites and requires only Mg²⁺ for activation. In our earlier studies, we found increased M-kinase activity in several MDR lines but were uncertain about its relevance [unpublished data]. In retrospect, this may also have been an atypical PKC isof orm like ζ or Λ. However, M-kinase enzyme may play a role that is yet undefined in MDR.

All of the work mentioned above has been in cell lines that have been drug-selected to produce the MDR phenotype in vitro. Intrinsic MDR and PKC have been studied only in human renal cell carcinoma cell lines which have an increased expression of PKC with concomitant increased expression of P-glycoprotein in lines with an increased MDR phenotype [43]. This important study, however, did not isolate and identify the specific PKC isoenzyme which is overexpressed in the more drug-resistant cell lines, but it does provide important preliminary evidence that PKC overexpression may be important to the mechanism of intrinsic MDR resistance. This area needs further investigation before conclusions can be attained.

**Phosphorylation of the P-Glycoprotein**

The P-glycoprotein is a phosphorylation substrate for a number of protein kinases other than PKC, including protein kinase A [44], PK-1 [45], and an unknown kinase which is GTP-regulated but not affected by inhibitors of cAMP, cGMP and PKC kinases [46]. It is theorized that phosphorylation of P-glycoprotein may regulate its function, but this has yet to be definitively proven. Evidence for the role of PKC phosphorylation which leads to P-glycoprotein functional alterations has been published from the laboratory of Dr. Robert Glazer, in which an alteration in the ATPase function of the P-glycoprotein and its ability to bind drugs in the transmembrane sites secondary to PKC phosphorylation are demonstrated [42]. Later in this paper we will present some preliminary evidence that PKC phosphorylation can affect both of these P-glycoprotein functions. In a recent paper from the laboratory of Higgins it was demonstrated that in HeLa cells, which have volume-activated chloride channels, PKC activation had no effect. If these cells were transfected with MDR1 and expressed P-glycoprotein, they then exhibited downregulation of volume-activated chloride channels with phorbol-induced PKC activation. Furthermore, if the serine sites for PKC phosphorylation in the linker region were mutated to alanine, the regulation of the ion channels by PKC activation was abolished. Alternatively, if serine phosphorylation sites were mutated to glutamate to mimic the negative charge from PKC phosphorylation on serine residues, this mimicked...
the effects of PKC activation on chloride channels [47]. Thus, at least in this model, this seems to be an unknown interaction between PKC phosphorylation and P-glycoprotein that alters volume-regulated Cl-channel activation. It is not known whether this model correlates with the MDR phenotype in cancer cells as of yet.

As previously stated, phorbol ester stimulation of P-glycoprotein phosphorylation in certain MDR tumor cell lines has been associated with an increase in the MDR phenotype. PKC-α transfection of the MDR1-transfected MCF-7 subclone BC-19 human breast cancer cells resulted in increased P-glycoprotein phosphorylation and increased drug resistance in MTT assays [38]. The amino acid sites of P-glycoprotein phosphorylation stimulated by phorbol in situ mimic the sites phosphorylated in isolated P-glycoprotein vesicles phosphorylated by PKC, which suggests that the same sites are phosphorylated on P-glycoprotein by PKC in vitro and in situ [24]. Also, basal phosphorylation of P-glycoprotein in KB-V1 cells is closely related to the same phosphorylation sites on P-glycoprotein obtained from phorbol ester stimulation in vitro [15, 24]. The amino acid sites phosphorylated by PKC are all serine residues (serine-661, -671 and one or more of serine-667, -675 and -683), which are present in the linker region between the two halves of the P-glycoprotein [15, 24, 28].

To study the role of PKC in phosphorylation and regulation of P-glycoprotein function, our laboratory has immunoprecipitated both P-glycoprotein and MARCKS protein from MDR MCF-7 cells labeled with P-32 and treated with MDR inhibitors. In these studies, phosphorylation of MARCKS was used as a marker of cellular PKC activity. Previously, enhanced phosphorylation of MARCKS was shown to correlate with enhanced expression of PKC-α in MCF-7 MDR cells [26], and PKC-βI in the fibroblast cell line R6-PKC-3 [41]. In situ phosphorylation experiments in which MDR MCF-7 cells were treated with the lysosphingolipid safingol, phosphorylation of P-glycoprotein and MARCKS protein was inhibited with similar concentration dependence. This leads to a decrease in the MDR phenotype in drug accumulation and MTT assays, suggesting that inhibition of PKC decreased P-glycoprotein activity. As already mentioned, the lysosphingolipid did not interact with vinblastine- or azidopine-binding sites and did not directly alter P-glycoprotein ATPase activity. The only effect found from the lysosphingolipid was decreased PKC activity, decreased MDR phenotype and phosphorylation of the P-glycoprotein [33].

To further study the role of PKC isoforms in the phosphorylation of P-glycoprotein, in vitro phosphorylation experiments with insect S9 MDR1-infected cells which expressed P-glycoprotein were phosphorylated by PKC isoforms and immunoprecipitated. P-glycoprotein was phosphorylated by all of the tested PKC isoforms which included α, β-I, II, γ, δ, ε, ζ and η (Fig. 1). These P-glycoprotein-expressing S9 cells contain very little endogenous PKC, which is a selective advantage for using this system. This characteristic is an advantage over membranes prepared from mammalian cells since phosphorylation of P-glycoprotein by endogenous PKC would complicate interpretation of PKC isoform phosphorylation. Experiments with PKC isoforms have also been performed to assess the effects of PKC phosphorylation on P-glycoprotein ATPase. Preliminary experiments showed that vinca alkaloid-stimulated ATPase activity of S9 MDR membranes was increased approximately twofold by prior phosphorylation of P-glycoprotein by PKC-α. In contrast, vinca alkaloid-stimulated ATPase activity was inhibited by approximately 50% with prior phosphorylation by PKC-βI and -βII isoforms. Furthermore, basal ATPase activity was not remarkably altered by the PKC isoforms. These studies suggest that phosphorylation of P-glycoprotein may be responsible for bidirectional regulation of drug-stimulated

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*Figure 1. Eight protein kinase-C isoenzymes were semipurified from baculovirus-infected SF9 insect cell systems. The human MDR1 P-glycoprotein, with a mutation of amino acid glycine 185 to valine, was also expressed in SF9 cells and used as membrane vesicles. Addition of PKC isoenzymes to P-glycoprotein vesicles was performed as previously described [26]. Phosphorylated P-glycoprotein was immunoprecipitated with C219 monoclonal antibody and analyzed on 7% SDS-PAGE and radiographed. The figure shows equal amounts of protein loaded into gels for a representative experiment. It demonstrates that all the PKC isoenzymes phosphorylate the P-glycoprotein, but differentially. The low mw of the P-glycoprotein (132 kDa) is due to lack of P-glycoprotein glycosylation in the insect cell system which does not affect its function.*
ATPase activity and may be the first example of divergent modulation of a physiological substrate by PKC isoenzymes. To test whether phosphorylation by these PKC isoenzymes affects drug binding, experiments were performed which tested the effects of PKC-βI phosphorylation on P-glycoprotein Sf9 MDR membranes. Autofluorograms demonstrated that prior phosphorylation by PKC-βI of P-glycoprotein membrane vesicles led to decreased azidopine photo-affinity labeling of P-glycoprotein. This suggested that phosphorylation by PKC-βI inhibited binding of azidopine to Sf9 P-glycoprotein. Thus, the effects of PKC-βI phosphorylation showed an inhibitory effect on ATPase activity and azidopine binding in these MDR1 Sf9 vesicles [48]. Although these experiments are still preliminary, in combination with previous work by Dr. Robert Glazer’s laboratory [42], they do suggest that PKC isoenzymes may alter the ATPase drug-stimulated activity and drug-binding abilities, dependent on which isoenzyme phosphorylates the P-glycoprotein.

More recently, work from Dr. Michael Gottesman’s laboratory has shown that the P-glycoprotein function is not altered when all the serine sites in the linker region of the P-glycoprotein are mutated to nonphosphorylatable amino acids [49]. This study does suggest some interesting possibilities: A) the P-glycoprotein is not modulated by phosphorylation in this particular cell type; B) differential phosphorylation of specific serine sites is required to modulate the P-glycoprotein which cannot be detected when all serine sites were concomitantly mutated, and C) when this finding is combined with our data, it suggests that the basal function of the P-glycoprotein may not be altered by PKC phosphorylation; rather, only drug- or phorbol ester-stimulated activity is altered.

To address the above possibilities, we have been investigating the preferential phosphorylation of P-glycoprotein with the synthesized P-glycoprotein-linker region peptide (termed PG-2) in collaboration with the laboratory of Dr. Tim Chambers. Preliminary data suggest that the calcium-dependent PKC isoenzymes, as well as the calcium-independent PKC isoenzymes, have a differential phosphorylation of specific serine sites in the linker region. Not only does PKC-β phosphorylate different sites than PKC-α, but the stoichiometry of phosphorylation is also different [50]. This preliminary work suggests that the phosphorylation of P-glycoprotein by PKC isoenzymes, which may lead to modulation of drug-stimulated P-glycoprotein function, is a complex biochemical process.

Another interesting (but yet unpublished) area of work involving PKC and P-glycoprotein function is the possibility that there may be cryptic sites of phosphorylation that only appear in the presence of drug substrate bound in the drug-binding regions of the transmembrane segments. This may lead to topographic changes which allow kinases like PKC to phosphorylate serine/threonine residues which may then allow further tertiary protein folding, due to increased net negative charges of the added phosphates, which may lead to alterations in ATPase activity and efflux. Similarly, phosphorylation of known or cryptic sites may also have untold effects which may not lead to changes in efflux. For instance, these are several reports in the MDR literature which describe altered compartmentalization of drugs as part of the MDR phenotype. Theoretically, evagination and vesicular/vacuolar formation of toxic drugs into compartments lined on the inside with inverted P-glycoprotein could keep drugs inside vesicles and pump ions in to acidify and further polarize these agents, resulting in sequestration of drugs which may be released later in less toxic form or into the extracellular space. Also, PKC phosphorylation may alter activation of the MDR1 promoter, leading to changes in P-glycoprotein content at the plasma membrane or intracellular vesicular membrane surface; if so, this possibility merits further study.

**SUMMARY**

There is ample evidence to suggest that PKC overexpression by one or more of its isoenzymes is associated with the acquired, drug-induced MDR phenotype. This appears to be cell type dependent as well as drug dependent in that doxorubicin-induced MDR seems to be more commonly associated with an overexpression of PKC-α isoenzyme. Another issue complicating this process is the involvement of other kinases which have not yet been fully explored. It is also possible that this is just a finding of association without a causal relationship in that doxorubicin-induced MDR may lead to other untold biochemical effects which upregulate PKC expression or downregulate PKC catalysis. Of particular interest is the direct correlation of increased PKC activity and content to the degree of intrinsic MDR in nondrug-exposed human renal cell carcinoma lines. To differentiate whether the association of PKC overexpression and MDR is an in vitro phenomenon only, further studies with intrinsic MDR cell lines are needed. Alternatively, the role of PKC upon P-glycoprotein induction via the MDR1 promoter is still largely unknown.

Preliminary data from our laboratory suggest that the PKC phosphorylation of the P-glycoprotein is a complex mechanism which involves differential phosphorylation of various serine residues in the linker region and cannot be easily explained, similar to the association of PKC upon volume-regulated Cl channels. It is also important to recognize the role of PKC in the antiapoptotic pathway which may further complicate the interpretation of results where low-level MDR is induced by PKC activation or by PKC transfection. Thus, it may be important for future studies to analyze markers for the anti-apoptotic pathway and to test for drug resistance to...
non-MDR associated drugs (i.e., 5-fluorouracil) which are associated with induction of apoptosis. Thus, the only thing that is truly clear is that this issue is still unclear and warrants further investigation to discern the actual importance of PKC phosphorylation of the P-glycoprotein in modulation of P-glycoprotein function.

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**ACKNOWLEDGMENTS**

Supported by NIH 5U01 CA46738 (RLF), ACS IRG 158J (RLF) and ARS DHP109 (TCC).

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