Vol. 169, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1990 Pages 253-259

CORRELATION OF PROTEIN KINASE C TRANSLOCATION, P-GLYCOPROTEIN PHOSPHORYLATION AND REDUCED DRUG ACCUMULATION IN MULTIDRUG RESISTANT HUMAN KB CELLS

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Received April 12, 1990

SUMMARY: Treatment of drug-resistant human KB carcinoma cells (KB-V1) with 0.2 μM phorbol 12-myristate 13-acetate (PMA) resulted in increases of 4-fold in both membrane-associated protein kinase C activity and phosphorylation of P-glycoprotein. The response was essentially complete after 30 min and was relatively stable, since both of these parameters remained elevated above basal levels in cells exposed to PMA for 24 hours. In contrast, long-term PMA treatment of drug-sensitive KB-3 cells caused complete depletion of protein kinase C. The rate of accumulation of [^{3}H]vinblastine in KB-V1 cells was 0.8 ± 0.1 pmol/mg/30 min in the absence, and 1.9 ± 0.2 pmol/mg/30 min in the presence, of 20 μM verapamil. Preincubation of cells with PMA resulted in a time-dependent decrease, up to 60% after 24 hours, in both of these values. These results suggest that protein kinase C mediated phosphorylation stimulates the drug transport activity of P-glycoprotein.

The development of resistance to chemotherapeutic agents is a significant limiting factor in the treatment of malignant tumors. One mechanism of resistance involves the amplification of the mdr multigene family which encodes a transmembrane protein of 150-170 KDa termed P-glycoprotein. This protein has a broad substrate specificity for the energy-dependent efflux of anticancer drugs from resistant cells [see (1,2) for recent reviews]. In multidrug-resistant cell lines, P-glycoprotein undergoes phosphorylation (3-7), and this post-translational modification is likely to be functionally important. In the multidrug-resistant human KB carcinoma cell line, KB-V1, phosphorylation is catalyzed by protein kinase C (PKC) (7), a Ca^{++}/phospholipid-dependent enzyme activated in cell membranes by

Abbreviations - PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate buffered saline; PMSF, phenylmethylsulfonyl fluoride; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle's medium; CHAPS, 3-[(3-cholamidopropyl) dimethyl-ammonio]1-propanesulfonate.

0006-291X/90 $1.50

diacylglycerol and tumor-promoting phorbol esters (8). Preliminary studies using KB-V1 cells indicated that phorbol ester treatment reduced verapamil-induced [^{3}H]vinblastine accumulation (7), similar to earlier results in drug-resistant human breast cancer cells (9). Taken together, these observations suggest the possibility that phosphorylation of P-glycoprotein may modulate its drug transport properties. Based on kinetic studies of the effects of phorbol ester on phosphorylation and drug accumulation, we show here that the activity of P-glycoprotein is stimulated by PKC catalyzed phosphorylation.

MATERIALS AND METHODS

Materials - [^{32}P]orthophosphate (8 mCi/ml), [gamma-^{32}P]ATP (3000 Ci/mmol) and [^{3}H]vinblastine (23 Ci/mmol) were obtained from Amersham and C219 monoclonal antibody, which recognizes P-glycoprotein (10), was from Centocor.

Cell Culture - Drug-sensitive KB-3 and drug-resistant KB-V1 cells were maintained in DMEM (4.5 g/l glucose) containing 10% fetal bovine serum and 10 mM Hepes, pH 7.3. KB-V1 cells were grown in the presence of 1 μg/ml vinblastine. For metabolic labeling with [^{32}P], cells in 160 cm^2 flasks were incubated for 4 hr in 10 ml of phosphate-free medium containing 0.8 mCi of [^{32}P]orthophosphate.

Preparation of Extracts and PKC Determination - Near-confluent cultures of cells in 160 cm^2 flasks were treated for various times with freshly prepared PMA dissolved in DMSO; controls received DMSO alone (0.1% v/v final concentration). Cells were gently scraped into ice-cold PBS, sedimented, and pellets suspended in 2 ml of buffer A (20 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 10 mM 2-mercaptoethanol, 1 mM EGTA, 1 mM EDTA and 1 mM PMSF). Cells were disrupted by nitrogen cavitation (400 p.s.i., 15 min), debris removed by centrifugation at 1,000 x g for 10 min, and a cytosolic fraction obtained by centrifugation at 100,000 x g for 1 hr. The membrane pellet was suspended in 1 ml of buffer A containing 1% w/v Triton X-100 and after 15 min on ice clarified by centrifugation (100,000 x g, 30 min). Samples were loaded onto 0.3 ml columns of DEAE-Sephacel equilibrated with buffer B (buffer A without sucrose), and, after washing with 5 ml buffer B, PKC was completely eluted with 2 ml 0.1 M NaCl in buffer B. PKC was assayed as described previously (11) by measuring incorporation of ^{32}P from [gamma-^{32}P]ATP to histone III-S in the presence of 0.5 mM $CaCl_2$, 50 μg/ml phosphatidyl-L-serine and 5 μg/ml diolein. Blanks, the values of which were subtracted, contained 1 mM EGTA instead of the cofactors. Protein was determined by the method of Bradford (12) using bovine serum albumin as a standard.

P-glycoprotein Phosphorylation - ^{32}P-labeled KB-V1 cells were treated with PMA for various periods, washed with ice-cold PBS, and membrane vesicles prepared as described previously (7). P-glycoprotein was solubilized by adding the detergent CHAPS to 1% w/v final concentration, as described (13). Immunoprecipitation with C219 monoclonal antibody and preparation of samples for SDS gel electrophoresis, performed using 6% acrylamide gels by the method of Laemmli (14), were carried out as detailed previously (7). Gels were stained with silver nitrate using reagents from BioRad Laboratories and quantitation of P-glycoprotein was performed by densitometric scanning of the gel. Radioactivity of excised bands was determined by scintillation counting.

Drug Accumulation Assays - KB-V1 cells in drug-free medium were plated at 3 x 10^5 cells/well in 24-well Costar plates. PMA (0.2 μm) was added to

appropriate wells. The next day, PMA was added to additional wells for various periods up to 2.5 hr. Then, 30 min prior to processing, the medium of each well was removed and replaced with 0.5 ml DMEM containing 50 mM Hepes, pH 7.35, 0.1 μCi [^{3}H]vinblastine, and, in some experiments, 20 μM verapamil. After 30 min incubation, the plates were washed three times in ice-cold PBS, the cells were detached with trypsin, and radioactivity determined (15). NaOH, 0.5 ml of 0.5 M, was added instead of trypsin to control groups of cells from each plate to determine protein content.

RESULTS

The kinetics of PMA-induced PKC translocation in KB-V1 cells are shown in Figure 1. The response, which was near maximal after stimulation for 30 min and relatively unchanged for periods of treatment up to 2.5 hr, resulted in translocation of approximately 50% of cytosolic PKC to the membrane. During this time, membrane-associated PKC specific activity increased 3- to 4-fold. Exposure of cells to PMA for 24 hr resulted in a return of cytosolic PKC activity to near basal level. Membrane PKC was decreased, relative to maximum levels obtained, but was still elevated relative to the basal level (Fig. 1). The results after 24 hr PMA treatment were unexpected since long-term exposure to PMA of numerous cell lines results in complete depletion, or down-regulation, of PKC (16,17). Further experimentation, using concentrations of PMA up to 1 μM, confirmed that PKC in KB-V1 cells is relatively resistant to PMA-induced down-regulation (Table I). In contrast,

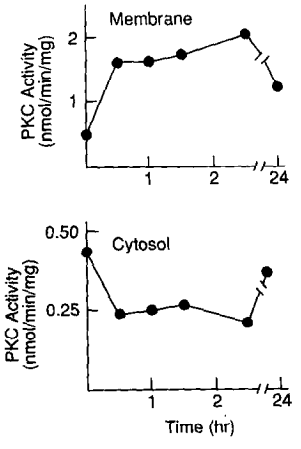


Figure 1. Time-course of PMA-induced PKC translocation in KB-V1 cells. Cultures of cells were treated with 0.2 μM PMA for the times indicated, cytosolic and membrane fractions prepared and partially purified by DEAE-Sephacel chromatography, and PKC activity determined, as described in "Materials and Methods."

Table I. Effect of short- and long-term exposure to PMA on activity and distribution of PKC in KB cells

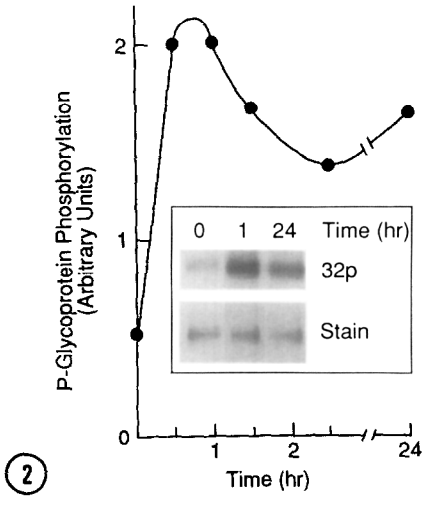
PMA Treatment		PKC Activity (% untreated)			
Duration	Concentration	KB-3		KB-V1	
(hr)	(μM)	Cytosol	Membrane	Cytosol	Membrane
0	0	100	100	100	100
0.5	0.2	11	293	56	342
24	0.2	11	0	74	192
24	1.0	10	0	59	133

Values given are averages of three independent experiments.

continuous exposure of drug-sensitive KB-3 cells to PMA resulted in characteristic depletion of PKC (Table I).

The kinetics of PMA-induced P-glycoprotein phosphorylation are shown in Figure 2. Maximum effects were observed after 1 hr and corresponded to a 4-fold increase in phosphate content. Phosphorylation levels declined with increasing times of exposure to PMA. This may be due to protein phosphatase activity associated with membranes from these cells (unpublished observation), since membrane PKC activity was relatively constant (Fig. 1). In cells treated with PMA for 24 hr, a relatively high level of phosphorylation of P-glycoprotein was maintained. Thus, in response to PMA, the increase in P-glycoprotein phosphorylation is of a magnitude similar to the increase in membrane PKC activity, and follows a similar time-course.

Accumulation of [^{3}H]vinblastine as a function of time of preincubation of KB-V1 cells with 0.2 μM PMA is shown in Figure 3A. The results are expressed as a percentage ratio of PMA-treated versus control values, since some variation in absolute values was observed in different experiments. Following a "lag" period of 30 min, a clear inverse relationship was observed between the amount of drug accumulated and the time of exposure of cells to PMA. [^{3}H]vinblastine accumulation was decreased 40% relative to control after preincubation with PMA for 2.5 hr, and decreased 60% after 24 hr. We next utilized verapamil, an inhibitor of drug transport (15), to induce higher levels of accumulation of [^{3}H]vinblastine, and examined the effect of PMA. The results, presented in Figure 3B, show that verapamil-induced



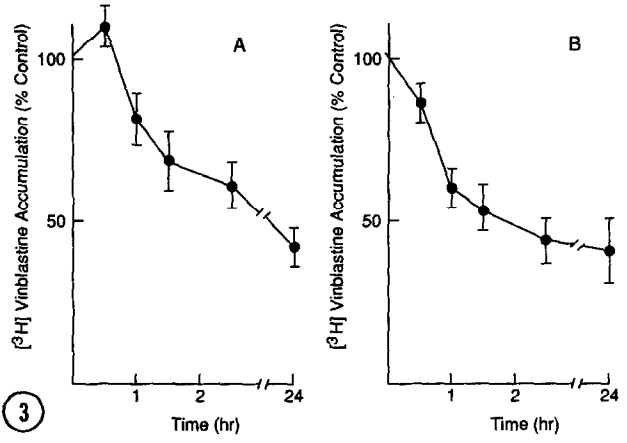


Figure 2. Time-course of PMA-induced P-glycoprotein phosphorylation. ^{32}P-labeled cells were treated with 0.2 μM PMA for the times indicated, membrane vesicles prepared, P-glycoprotein immunoprecipitated, and samples analyzed by 6% acrylamide SDS gel electrophoresis, silver staining and autoradiography. Quantitation of P-glycoprotein phosphorylation was based on comparison of radioactivity and amount of protein as described in "Materials and Methods." The inset shows typical results obtained after 0, 1 and 24 hr treatment with PMA.

Figure 3. Effect of PMA on [^{3}H]vinblastine accumulation. KB-V1 cells were preincubated with 0.2 μM PMA for the times indicated and 0.1 μCi [^{3}H]-vinblastine added alone (A) or with 20 μM verapamil (B). After an additional 30 min, cells were washed and radioactivity and protein determined, as described in "Materials and Methods." In (A), 100% = 0.785 pmol/mg; in (B), 100% = 1.895 pmol/mg. Results are expressed for each time point as [mean (PMA)/mean (control)] x 100, n=4. The magnitude of the error bar at each time point was calculated from $\sqrt{[S_1^2 + S_2^2]}$, where S_1 = S.E. (control) and S_2 = S.E. (PMA), n=4.

[^{3}H]vinblastine accumulation was reduced significantly by PMA treatment in a time-dependent manner, similar to results of Fig. 3A. However, in this case, no lag period was observed, and the effect of PMA was more pronounced at shorter times of exposure. It should be noted that under the conditions used, verapamil did not affect phosphorylation of P-glycoprotein (7).

Dose-response curves showed that the maximal effect of PMA on drug accumulation was observed at concentrations of 0.1 - 0.5 μM. Additionally, in contrast to PMA, the inactive analog phorbol 12,13-diacetate had no significant effect on drug accumulation.

DISCUSSION

To examine the role of P-glycoprotein phosphorylation, catalyzed in KB-V1 cells by PKC (7), the effect of PMA on [^{3}H]vinblastine accumulation was

examined. Here we show that drug accumulation, an accurate and biologically relevant measure of the activity of P-glycoprotein, is significantly reduced by preincubation of the cells with PMA. Both basal and verapamil-induced [^{3}H]-vinblastine accumulation are decreased. Under the same conditions, PMA induces membrane association, and thus activation, of PKC, resulting in significantly increased phosphorylation of P-glycoprotein. Thus, the ability of P-glycoprotein to transport drugs appears to be stimulated by phosphorylation catalyzed by PKC. Whereas stimulation of transport appeared directly related to time of exposure to PMA, the phosphorylation of P-glycoprotein was maximal after 1 hour, and then reduced. While this may indicate a non-catalytic effect of phosphorylation on activity, measurement of net phosphorylation level does not take into account the possibility of differential phosphorylation at multiple sites. Also, phosphorylation may promote a more active conformation of the protein, not readily reversed by dephosphorylation.

To further define the role of PKC-catalyzed phosphorylation in P-glycoprotein function, it will be necessary to inhibit phosphorylation, and studies utilizing PKC inhibitors are in progress. An alternative approach, where PKC is depleted by prolonged exposure of cells to PMA, was precluded since KB-V1 PKC was relatively resistant to PMA-induced down-regulation (Table I). This property of KB-V1 cells, which is unusual but not unique (16,17), was not exhibited by KB-3 cells. This phenomenon thus appears to be related to the development of drug resistance in this cell line, and hence, merits further investigation.

Evidence for a regulatory role of PKC in numerous secretion and transport processes has been documented. PKC activation is associated with release of neurotransmitters, hormones, enzymes and other agents, and with transport of glucose and ions such as Ca^{++}, H^+, Na^+, K^+ and Cl^- (8,18). The transporter proteins responsible for some of these processes have been identified and in some cases phosphorylation <u>in vivo</u> demonstrated, e.g., glucose transporter (19) and Na^+/H^+ antiporter (20). However, to our knowledge, there are no

reports in which a change in transport activity has been correlated with PKC catalyzed phosphorylation of specific transporters. The results presented here suggest a key role for PKC in phosphorylation and regulation of the multidrug transporter, a protein which shares significant structural homology with other transporters (21,22; see also 23).

ACKNOWLEDGMENTS

The authors are grateful to Drs. M. Gottesman and I. Pastan for kindly providing the cell lines used in this study and to Dorothy Bonenberger for typing the manuscript.

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