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PROTEIN KINASE C DESENSITIZATION BY PHORBOL ESTERS AND ITS IMPACT ON GROWTH OF HUMAN BREAST CANCER CELLS

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Active phorbol esters such as TPA (12-O-tetra-decanoyl-phorbol-13-acetate) inhibited growth of mammary carcinoma cells (MCF-7 > BT-20 > MDA-MB-231 > = ZR-75-1> HBL-100) with the exception of T-47-D cells presumably by interacting with the phospholipid/Ca^{2+}-dependent protein kinase (PKC). The non-responsive T-47-D cells exhibited the lowest PKC activity. A rapid (30 min) TPA-dependent translocation of cytosolic PKC to membranes was found in the five TPA-sensitive cell without affecting cell growth. However, TPA-treatment of more than 10 hours inhibited reversibly the growth of TPA-responsive cells. This effect coincided with the complete loss of cellular PKC activity due to the proteolysis of the translocated membrane-bound PKC holoenzyme (75K) into 60K and 50K PKC fragments. Resumption of cell growth after TPA-removal was closely related to the specific reappearance of the PKC holoenzyme activity (75K) in the TPA-responsive human mammary tumor cell lines suggesting an involvement of PKC in growth regulation.

Phorbol esters such as TPA are known to bind specifically to protein kinase C (PKC) and to stimulate the enzyme (1-4). These compounds are able to substitute for diacylglycerol (DG) which is generated during the inositol phospholipid breakdown in response to various hormonal stimuli (4,5). An important clue to the mode of action of tumor promoters is that they bypass the DG-dependent physiological regulatory mechanisms resulting in a permanent activation of PKC (3-5). This mechanism explains in part their effects on a variety of cellular processes such as growth arrest and differentiation (6,7). The role of PKC in TPA-dependent growth-inhibition was studied in

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a set of mammary carcinoma cells exhibiting different sensitivities towards tumor promoters (8,9).

This study gives strong evidence that TPA mediates a rapid translocation of cytosolic PKC to the membranes followed by its proteolytic degradation. The proteolysis of the PKC holoenzyme appears to be closely related to the growth inhibition of breast cancer cells.

MATERIALS AND METHODS

Materials and chemicals were obtained from the following companies: Tissue culture dishes: FALCON, Cockeysville, MD. Sera and culture media: GIBCO. Fatty acid free bovine serum ambumine (BSA-FAF), lysine-rich histone H1 (calf thymus type V-S), 1,2-diolein L-α-phosphatidyl-L-serine, leupeptin and 4 β-phorbol-12-myristate-13-acetate (TPA): SIGMA, St. Louis, MO. and glass fiber filters (GF/C): WHATMAN, Maidstone, Great Britain; γ(^{32}P)ATP (S.A. 10 Ci/mmol): NEN, Boston, MA. Reagents for protein determination: BIO-RAD, Munich, FRG; acrylamide, N,N,N'N'-tetra methylethylenediamine (TEMED) N,N'-methylene-bisacrylamid (BIS): SERVA, Heidelberg, FRG; Millipore filters (PHWP 0.45 um): MILLIPORE, Bedford, MA.

Cell culture conditions

The mammary carcinoma cell lines MCF-7, T-47-D, ZR-75-1, MDA-MB-231, HBL-100 and BT-20 were obtained from the Mason Research Institute (Rockville, MD). The cell lines were routinely grown and growth experiments were carried out as reported elswhere (8,9).

Preparation of cytosol and crude membrane fractions

Cells were disrupted by sonication at 4°C in ice-cold 20mM Tris-HCl, pH 7.4, 2mM EGTA, 2mM EDTA, 6mM β-mercaptoethanol, 20 μg/ml leupeptin and 2 μg/ml aprotinin; cytosol and membrane extracts were prepared exactly as described previously (2).

Protein kinase assays

PKC activity was assayed after polyacrylamide gel electrophoresis (PAGE) by measuring ^{32}P incorporation from γ-(^{32}P)ATP into lysine-rich histone (type V-S: SIGMA) in the presence of 300 μM Ca^{2+} and phospholipids (100 μg phosphatidylserine, 10 μg diolein as reported earlier (10). Alternatively PKC activity was assayed in absence of Ca^{2+} and phospholipids but with protaminesulfate as exogeneous substrate (11). cAMP-dependent protein kinase activity (PKA) was measured as described before (12).
One unit of protein kinase activity is defined as the amount of enzyme transferring one pmole of γ-(^{32}P)ATP to exogeneous substrate (histone or protaminesulfate) during 1 min under optimal assay conditions.

Polyacrylamide gel electrophoresis (PAGE)

Lower (resolving) gels were polymerized at total monomer concentrations between 4.5 - 12% whereas upper (stacking) gels

were prepared at a 3.5% total gel concentration using the system B (13, 14). After electrophoresis PKC activity was assayed quantitatively (10).

Quantitative PAGE

The Ferguson plot i.e. the weihgted linear regression of 10g Rf versus total gel concentration, its negative slope (K_R), the ordinate intercept (Y_0) and the joint 95% confidence envelopes of K_R and Y_0 of PKC activities as well as the conversion of K_R into molecular weights (MW) were computed according to RODBARD and CHRAMBACH (14).

Other analytical methods

Protein was determined by the method of BRADFORD (15) using the Bio-Rad reagents and BSA as standard. Statistical significance was analyzed by the Wilcoxon rank sum test.

RESULTS AND DISCUSSION

TPA inhibited growth of the six breast cancer cell lines investigated to various degress (8,9); the most inhibited was the MCF-7 cell line whereas growth of the T-47-D cells was not affected by the tumor promoter (Table 1). In contrast to the total PKA activities (cytosolic + membrane-bound) the total PKC levels varied widely among the cell lines (Table I). Cellular PKC activity was in MDA-MB-231 cells the highest (2323 units/mg), while the T-47-D cells exhibited extremely low levels (35 units/mg).

TABLE 1: TOTAL PKC ACTIVITIES AND TPA-DEPENDENT GROWTH-INHIBITION

CELL LINE	TOTAL PROTEIN KINASE ACTIVITY units/mg of protein		TPA-DEPENDENT GROWTH INHIBITION (% of control)
	PKC	PKA	
MCF-7	616 ± 23	292 ± 34	100
ZR-75-1	201 ± 11	228 ± 8	45
T-47-D	35 ± 16	276 ± 75	0
MDA-MB-231	2323 ± 196	205 ± 54	50
HBL-100	2118 ± 336	282 ± 46	40
BT-20	873 ± 58	282 ± 73	70

Subcellular fractions were prepared from each human mammary cell line and analyzed for PKC and PKA activities as reported in methods. The total activity consists of the cytosolic and membrane associated protein kinase activities. Results are expressed as mean ± SD of three independent experiments. Growth experiments were conducted as described in methods. Cells were incubated with 100nM TPA for 72 hours before DNA determinations are expressed as % inhibition of control (8).

Treatment of intact cells with 100nM TPA resulted in a time-dependent transfer of cytosolic PKC activity to the respective membrane fractions (Fig. 1). This effect was specific for TPA and did neither affect the subcellular distribution nor the total PKA activity of TPA-treated cells (9). In the cytosolic as well as in the membrane fraction three protein kinase activities (I - III) were resolved by PAGE after TPA treatment (Fig. 2B). The major protein kinase activity peak (I) was phospholipid/Ca^{2+}-dependent and represented the PKC holoenzyme (10). The two minor activities (II and III) both independent of Ca^{2+}/phospholipids were recognized as proteolytic fragments of PKC, since only present in TPA-treated cells (Fig. 2B) and prefering protaminsulfate as substrate (11,16-18). These PKC activities (I,II and III) were physically different on quantitative PAGE as evidenced by the fact that their

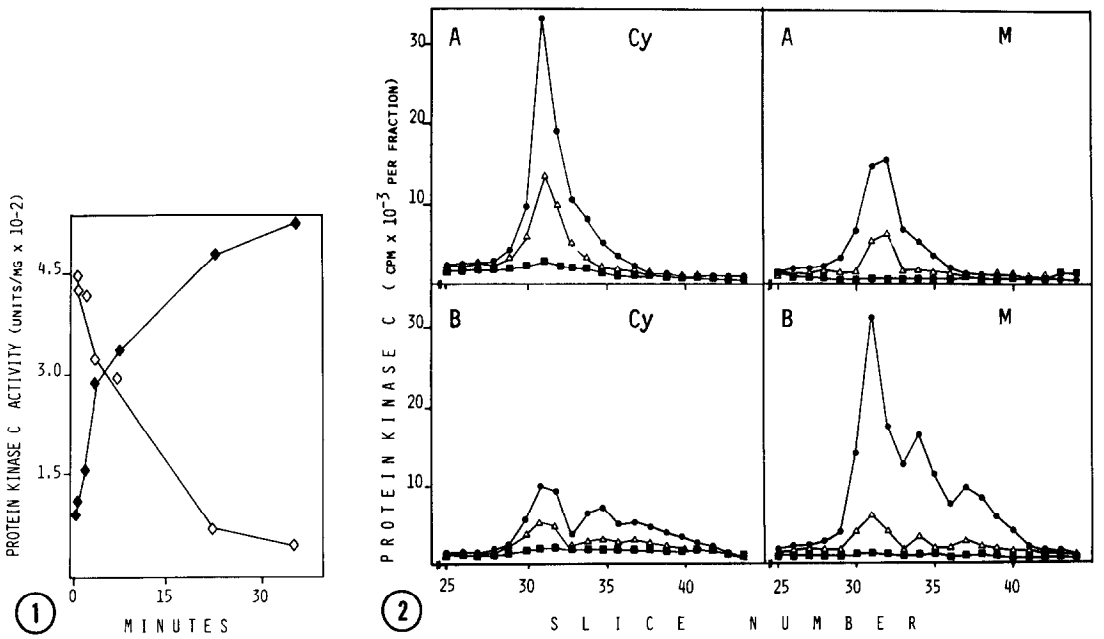

FIGURE 1. Time course of TPA-effect on PKC activity: Monolayer cultures of MCF-7 cells were incubated with 100nM TPA at 37°C. At indicated times cytosol and membrane extracts were prepared and quantitated for PKC activity as described in methods. Each point is the mean ± SD of triplicate determination: (◇) cytosolic and membrane-bound PKC (◆).

FIGURE 2. PKC activities analyzed by PAGE without (A) or with TPA: Monolayer cultures of MCF-7 cells were incubated without (A) or with 100nM TPA for 30 min (B). Cytosols(Cy) and membrane (M) fractions were prepared and analyzed by PAGE at a gel concentration of 6% as described in methods. Protein kinase activity in the presence (△) or absence of Ca^{2+} and phospholipids (■) or with protaminesulfate as substrate (●).

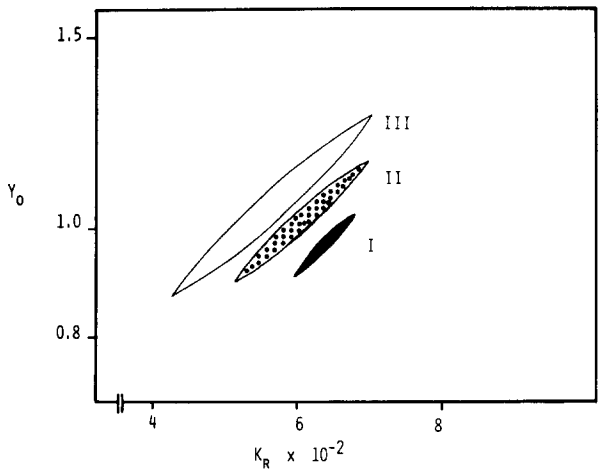

FIGURE 3. Comparison of physical characteristics of membrane-bound PKC activities (I, II, III) of TPA-treated MCF-cells: Molecular characterization of membrane-bound PKC activities (following TPA treatment of intact MCF-7 cells as described in Fig.2) in terms of their joint 95% confidence limits of Kr and Y_0 were performed by quantitative PAGE as described in methods.

joint 95% confidence envelopes of K_R and Y_0 (14) did not overlap (Fig.3).

Estimation of molecular weights by quantitative PAGE (Table 2) demonstrated that the PKC holoenzyme (I) had a MW of 75K; the proteolytic fragments exhibiting MWs of 60K (II) and 50K (III) respectively (Table 2). In untreated mammary cells only the 75K PKC was resolved by PAGE in the cytosol as well as in the membrane fractions (Fig. 2A). It appears that TPA not only induces a rapid integration of PKC into membranes (17, 19) but also enhances the activity of a membrane-bound protease and/or its translocation from cytosol

TABLE 2: PHYSICAL CHARACTERIZATION OF MEMBRANE-BOUND PKC ACTIVITIES (I,II,III) OF TPA-TREATED MCF-7 CELLS BY QUANTITATIVE PAGE

PKC ACTIVITIES	$K_R \times 10^{-2}$	$sK_R \times 10^{-2}$	$MW \times 10^{-3}$
PKC-holoenzyme (I)	6.54	0.26	75
PKC-activity II	6.10	0.29	61
PKC-activity III	5.73	0.42	52

Intact MCF-7 cells were incubated for 30 min with 100nM TPA. The membrane fraction was prepared and PKC activities were analized by quantitative PAGE as described in methods. The physical parameters of size (K_R) and the estimation of MWs were determined by quantitative PAGE as described (10, 14).

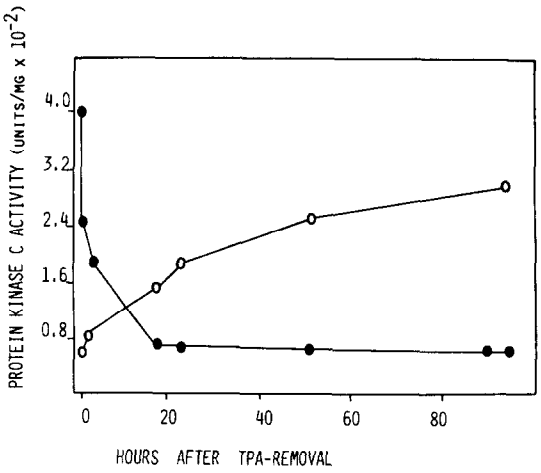

FIGURE 4. Effect of TPA-removal on PKC activity after short-term TPA-treatment (30 min): Intact MCF-7 cells were treated for 30 min with TPA as described in Fig.2, subsequently the medium was replaced with fresh regular growth medium (without TPA). At indicated times cytosol (○) and membrane (●) were prepared and quantitated for PKC activity as described in methods. Each point represents mean ± SD of triplicate determinations.

to membranes leading to the proteolysis of the membrane-associated PKC-holoenzyme (16-19). Removal of TPA after 30 min resulted in a slow decline of membrane-associated PKC activities (75K, 60K and 50K) paralleled by a slow increase of the cytosolic PKC-holoenzyme activity (75K only, Fig. 2) returning to control levels of PKC-holoenzyme activities in both cytosol and membrane fractions within 15 and 90 hours respectively (Figs.2,4). One has to point out that incubation of 30 to 60 min with 100nM TPA did not inhibit growth of breast cancer cells. These three PKC activities (I-III) were found always during or after short-term TPA-treatment (Figs. 2 and 4). Of interest is that the TPA-dependent PKC translocation was observed only in the five TPA-sensitive cells whereas in the TPA-unresponsive T-47-D cell, which exhibited only extremely low levels of PKC activity, such an analysis was excluded. Prolonged incubation with 100nM TPA resulted in a gradual loss of membrane-bound PKC-holoenzyme activity in all breast cancer cell lines (Fig. 5B). Complete loss of the PKC-holoenzyme (75K) was noted after two hours which was followed by a slower but total disappearance of the 60K (II) and 50K (III) PKC activities within 12 hours (Fig. 5B). No PKC activity was detectable after 12 hours in breast cancer cells (Fig. 5B) and coincided with the minimal incu-

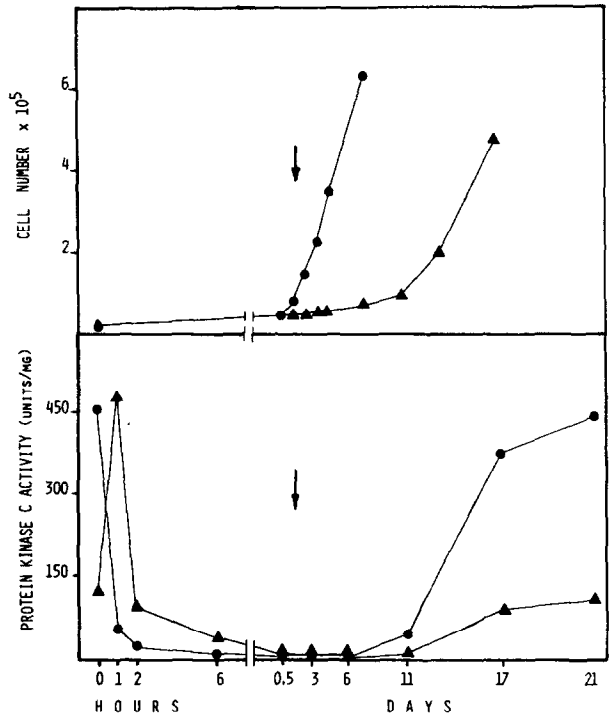

FIGURE 5. Effect of long-term TPA-treatment on PKC activity and on growth of MCF-7 cells: MCF-7 cells were incubated for 72 hours with or without 100nM TPA. After this period TPA was removed and the incubation was continued in regular growth medium.
(A): At indicated times the cell numer were counted TPA-treated cells (▲), control cells (●).
(B): Parallel TPA-treated cultures were quantitated for membrane-bound (▲) and cytosolic (●) PKC activities as described in methods.

bation time required to induce inhibition of growth (Fig. 5A).The cells remained growth-inhibited for 5 to 20 days after TPA-removal depending on the incubation time and the concentration of TPA used (Fig. 5A). Concomitant with the resumption of growth the PKC-holoenzyme activity (75K) increased with a standard subcellular distribution (9) and returned to control levels when cells achieved their normal growth rates (Fig. 5A).

There was no differences in cAMP-dependent protein kinase (PKA) activities during the incubation with TPA and after its removal nor in the overall cellular protein synthesis as determined by pulse labelling with ^{35}S-methionine (date not shown) (20). TPA is known to induce a specific desensitization of the phorbol-ester receptor (21-23) and to have little effect on protein-synthesis of human mammary tumor cells (24). Therefore it

appears that the desensitization of the phorbol-ester receptor occurs via the proteolysis of the PKC-holoenzyme result. This loss of phorbol-ester receptor binding capacity appears to correlate with the growth inhibition of TPA-sensitive breast cancer cell lines. It is possible that the very low levels of PKC of T-47-D cells is responsible for its unresponsivness towards TPA. These findings suggest a relationship between TPA-response and quantity of PKC activity and indicate a specific involvement of PKC in the TPA-mediated growth-inhibition of breast cancer cells.

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