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Continuous synthesis of two protein kinase C-related proteins after down-regulation by phorbol esters

(breast cancer/phorbol ester/proliferation/protein kinase C/antibody)

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ABSTRACT **The phorbol 12-myristate 13-acetate (PMA)-dependent down-regulation of immunoprecipitable protein kinase C was studied in human breast cancer cell lines that display different growth inhibitions toward the tumor promoter. PMA induces translocation of [^{35}S]methionine-prelabeled cytosolic protein kinase C to membranes, followed by complete degradation of the enzyme ($t_{1/2}$, 2 hr). PMA does not affect the protein kinase C synthesis; 20–80% of total protein kinase C of control cells was still immunoprecipitable as membrane-bound 74- and 80-kDa protein kinase C-related polypeptides if cells were allowed to incorporate [^{35}S]methionine during PMA exposure for >6 hr. These two proteins lack protein kinase activity and phorbol ester binding but reveal V8 peptide patterns identical to the active forms of protein kinase C (77/80 kDa) of PMA-untreated cells. The amounts of the immunoprecipitable membrane-bound 80-kDa protein kinase C-related polypeptide synthesized during the prolonged PMA treatment appear to inversely correlate with the extent of PMA-mediated growth inhibition of the respective human breast cancer cell line. These data suggest that after homologous down-regulation, functional protein kinase C (77/80 kDa) is replaced by a population of membrane-associated but enzymatically inactive protein kinase C-related polypeptides (74/80 kDa).**

Tumor-promoting phorbol esters evoke pleiotropic responses in various tissues and cells (1–4). They bind to high-affinity phorbol ester receptors, which have been identified as members of the multigene family of phospholipid and Ca^{2+}-dependent protein kinases (protein kinase C) (5–9). Protein kinase C is involved in signal transduction by coupling receptor-mediated inositol phospholipid turnover with a variety of cellular functions (10, 11). Hormones and growth factors that generate diacylglycerol lead to a transient translocation of cytosolic protein kinase C to membranes, which apparently reflects the intracellular activation of the enzyme (12–14). However, tumor promoters permanently activate and dock protein kinase C to membranes, bypassing the agonist-mediated increase of diacylglycerol (15). The exposure of estrogen receptor-containing (ER+) and estrogen receptor-lacking (ER−) human breast cancer cell lines to phorbol 12-myristate 13-acetate (PMA) leads to a nontoxic and reversible inhibition of cell proliferation (16). The time course of PMA-dependent translocation and subsequent down-regulation of protein kinase C is similar in all cell lines, although the individual cells are differently growth-inhibited by tumor promoters (17). These data indicate that the molecular mechanism(s) responsible for the individual growth responses toward PMA may reside distal to the down-regulation of protein kinase C.

The present study demonstrates that during prolonged PMA treatment the active membrane-bound 80-kDa protein kinase C holoenzyme is replaced by two immunoprecipitable protein kinase C-related polypeptides of 74 and 80 kDa that lack protein kinase activity and phorbol ester binding. A possible role of these polypeptides in the growth response of human breast cancer cells to PMA is suggested.

MATERIALS AND METHODS

Cell Culture. Cells were cultured as described (18). The MCF-7-CG and MCF-7-DF are variants of MCF-7 cells (Mason Research Institute, Rockville, MD).

Determination of Protein Kinase C Activity and Phorbol Ester Binding. Subcellular fractionation, protein kinase C activity, and phorbol ester binding by ^{3}H-labeled phorbol 12,13-dibutyrate of cytosols and membranes were performed and quantified as described (17, 19, 20).

Purification of Protein Kinase C and Production of Anti-Protein Kinase C Antibodies. Purification of protein kinase C from pig brain and generation of polyclonal antiserum are described elsewhere (21, 22). Antiserum was affinity-purified exactly as described (23) and is referred to as the anti-protein kinase C antibody. Antiserum 0442 raised against the amino acid sequence 280–292 of bovine brain protein kinase C (7) was a generous gift of P. J. Parker (Ludwig Institute, Imperial Cancer Research Fund, London).

Immunoprecipitation. Cells (2–5 × 10^{7}) were labeled for 6 hr at 37°C with 100 μCi of [^{35}S]methionine per ml (Amersham; 1200 Ci/mmol; 1 Ci = 37 GBq) in methionine-free Dulbecco's minimum essential medium (Amimed, Basel) supplemented with 1 μM unlabeled methionine. PMA (Sigma) was added either before, during, or after the labeling. Cells were fractionated with or without phosphatase inhibitors (50 mM NaF/100 μM Na_3VO_4) as described (17). Equal amounts of cytosol and membranes were incubated for 3 hr at 20°C with anti-protein kinase C antibody or antiserum 0442 (with or without 1 μg of unlabeled antigen) in 20 mM Tris·HCl, pH 7.4/0.5% sodium deoxycholate/0.5% Triton X-100/0.1% $NaDodSO_4$/1.5 mM iodoacetamide/2.5 mg of ovalbumin per ml/5 mM EGTA/1 mM EDTA/50 mM NaF/100 μM Na_3VO_4/200 μM leupeptin/600 nM aprotinin. Immunocomplexes were precipitated with 100 μl of protein A-Sepharose CL-4B (50%) and washed five times in RIPA buffer (0.15 mM NaCl/0.05 mM Tris·HCl, pH 7.2/1% Triton X-100/1% sodium deoxycholate/0.1% $NaDodSO_4$). Immunoprecipitated protein kinase C was analyzed on 8% $NaDodSO_4$ gels (24), subjected to fluorography (25), and quantified (22).

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Abbreviations: PMA, phorbol 12-myristate 13-acetate; ER, estrogen receptor.

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V8 Peptide Mapping. Limited proteolysis was performed exactly as described (26). Radiolabeled protein kinase C bands were excised, rehydrated, and electrophoresed for 16 hr at 40 V in the presence of 0.1 μg of *Staphylococcus aureus* V8 protease on a $NaDodSO_4$ gel consisting of 5% (stacking) and 15% (separating) acrylamide.

Autophosphorylation of Purified Protein Kinase C. Purified protein kinase C was autophosphorylated for 10 min at 32°C in 20 mM Tris·HCl, pH 7.4/10 mM $Mg(NO_3)_2$/10 μM [γ-^{32}P]-ATP (5 μCi) with or without 100 μg of L-α-phosphatidyl-L-serine/1 μM PMA and analyzed on $NaDodSO_4$/polyacrylamide gel or immunoprecipitated.

Other Procedures. Protein was determined by the method of Bradford (27) using the Bio-Rad reagents. Statistical significance was analyzed by the Wilcoxon rank-sum test.

RESULTS

Characterization of the Anti-Protein Kinase C Antibody. About 1 μg of highly purified protein kinase C with a molecular mass of 77 kDa was obtained from 1 g of porcine brain (Fig. 1*A*). Immunoprecipitation of autophosphorylated purified protein kinase C resulted in a single band of 80 kDa (Fig. 1*B*). However, MDA-MB-231 cells labeled with [^{35}S]methionine exhibited two forms of immunoprecipitable protein kinase C, a cytosolic 77-kDa form and a membrane-bound 80-kDa form (Fig. 1*C*). A shift of immunoprecipitable cytosolic protein kinase C from 77 to 80 kDa was observed if cytosols were prepared in the presence of phosphatase inhibitors or $NaDodSO_4$ (Fig. 1*C*).

Protein Kinase C Levels of Human Breast Cancer Cells. The cellular levels of immunoprecipitable protein kinase C correlated with the amounts estimated by immunoblotting (22), enzyme activity, and phorbol ester binding (Fig. 2). All cell lines incorporated similar levels of [^{35}S]methionine (data not shown). In general, ER− cells displayed significantly ($P < 0.001$) higher amounts of protein kinase C as compared to ER+ cells (Fig. 2).

Effect of PMA on Immunoprecipitable Protein Kinase C of MDA-MB-231 Cells. The half-life of immunoprecipitable protein kinase C was similar in all cells ($t_{1/2}$, >24 hr) (Fig. 3*A*). PMA treatment did not affect the incorporation of [^{35}S]methionine into total cellular protein (data not shown) but induced a time-dependent translocation of [^{35}S]methionine-prelabeled cytosolic protein kinase C into the corresponding

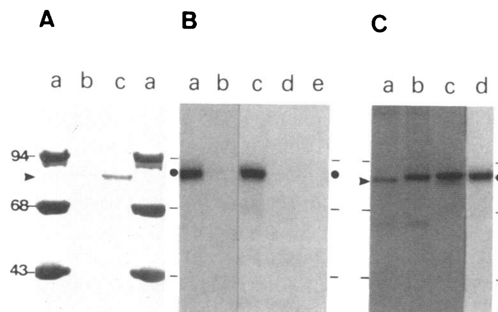

FIG. 1. Characterization of the anti-protein kinase C antibody. (*A*) Coomassie blue-stained gel: 0.5 μg (lane b) and 3 μg (lane c) of electroeluted protein kinase C. Lanes a, size markers. (*B*) Autoradiograph of protein kinase C autophosphorylated *in vitro* in the presence (lane a) or absence (lane b) of phosphatidylserine/PMA; immunoprecipitation of autophosphorylated protein kinase C (lane c), competed with pure antigen (lane d) or preimmune serum (lane e). (*C*) Immunoprecipitation of [^{35}S]methionine-labeled protein kinase C of MDA-MB-231 cells; cytosol prepared in the absence (lane a) or presence (lane b) of phosphatase inhibitors or $NaDodSO_4$ (lane c); membranes (lane d). Arrowheads, 77-kDa protein kinase C; solid circles, 80-kDa protein kinase C. Numbers on left are kDa.

membranes (Fig. 3*B*). During this rapid PMA-dependent subcellular redistribution (30 min), no loss of immunoprecipitable protein kinase C was observed (Figs. 3*B* and 4 *A* and *B*), although total enzyme activity decreased by 40–60% (Fig. 4*C*). Translocation of immunoprecipitable protein kinase C by PMA was independent of the [^{35}S]methionine labeling protocol.

Prolonged PMA treatment (>40 min) resulted in a gradual loss of [^{35}S]methionine-prelabeled membrane-bound protein kinase C, which paralleled the loss of the membrane-associated enzyme activity ($t_{1/2}$, 2 hr) (Figs. 3*B* and 4*C*). A similar rate of down-regulation was observed if cells were allowed to incorporate [^{35}S]methionine during the PMA treatment (Figs. 3*B* and 4*B*). In addition, proteins of ≈50 kDa were specifically immunoprecipitated from PMA-treated membranes (Fig. 4*A*). Interestingly, enhanced immunoprecipitation of a cytosolic 150-kDa protein was noted in ER− cell lines with increasing time of PMA treatment (Fig. 4*A*). This protein seems to share antigenic epitopes with protein kinase C (Fig. 4*A*, compare lane f with lane a).

Immunoprecipitable Protein Kinase C-Related Polypeptides After Down-Regulation by PMA. No immunoprecipitable protein kinase C (80 kDa) was detectable in [^{35}S]methionine-prelabeled MDA-MB-231 cells after 6–10 hr of PMA treatment (Fig. 3*B*), which is in agreement with the total loss of enzyme activity (Fig. 4*C*) and phorbol ester binding (data not shown). However, exposure of unlabeled cells to PMA for 6–120 hr, followed by [^{35}S]methionine labeling during the last 6 hr, revealed two immunoprecipitable 74- and 80-kDa protein kinase C-related polypeptides exclusively associated with membranes (Fig. 4*A*). According to this finding, the active membrane-bound 80-kDa protein kinase C seems to be replaced by two enzymatically inactive 74- and 80-kDa protein kinase C-related polypeptides (Fig. 4). Antiserum 0442 also immunoprecipitated a membrane-associated 74/80-kDa doublet of long-term PMA-treated as well as the functional 77/80-kDa protein kinase C forms of untreated MDA-MB-231 cells (Fig. 5). According to the V8 peptide mapping, no major differences were evident in the peptide pattern of the enzymatically active (77/80 kDa) and inactive (74/80 kDa) protein kinase C forms (Fig. 6).

These data strongly indicate that human breast cancer cells synthesize membrane-bound protein kinase C-related proteins (74/80 kDa) in the presence of PMA. Partially purified 74- and 80-kDa protein kinase C-related proteins did not display any protein kinase C activity with either of the substrates tested (histones H1, H2A, H2B, H3, H4, HIII-S, protamine, kemptide, vinculin) (data not shown).

Synthesis of Protein Kinase C-Related Polypeptides in Long-Term PMA-Treated Human Breast Cancer Cells. The immunoprecipitable inactive membrane-bound 74/80-kDa protein kinase C-related doublet was found in all cell lines tested after PMA-dependent down-regulation of the active forms of the kinase (77/80 kDa). However, the amounts of the 74/80-kDa proteins varied widely among the cell lines if treated with 300 nM PMA for 48 hr (Fig. 7): T-47-D and rat pituitary GH_3 cells, which are not growth-inhibited by PMA, revealed ≈80% immunoprecipitable membrane-bound 80-kDa protein kinase C-related polypeptide and negligible amounts of the 74-kDa protein as compared to control cells (Fig. 7). In contrast, the membrane fractions of MDA-MB-231, MCF-7-DF, and BT-20 cells showed variable amounts of immunoprecipitable 74/80-kDa protein kinase C-related polypeptides after down-regulation of protein kinase C activity by PMA (Fig. 7). The levels of the immunoprecipitable 80-kDa protein kinase C-related polypeptide appear to correlate inversely with the extent of growth inhibition induced by PMA in these cell lines (Fig. 7).

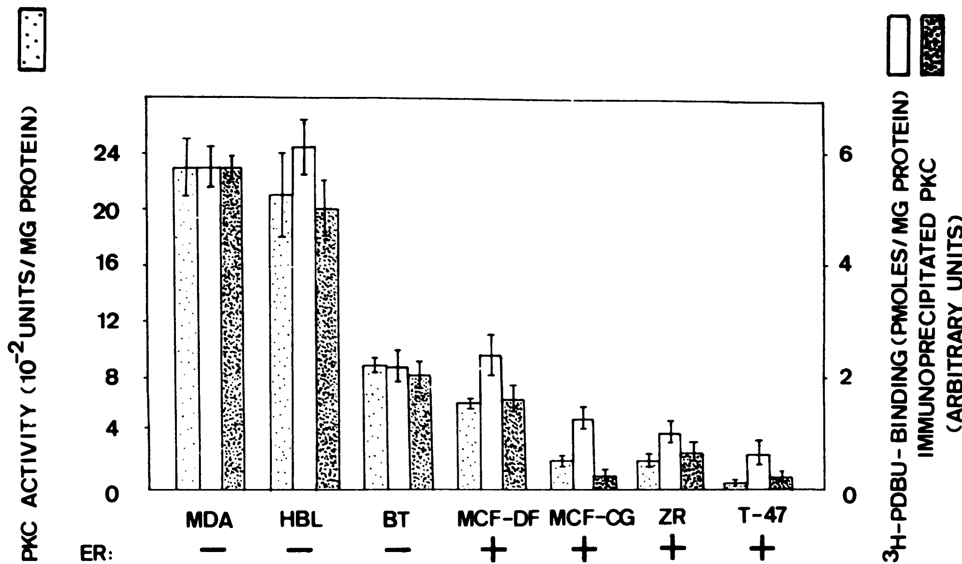

FIG. 2. Quantification of protein kinase C (PKC) in human breast cancer cells. Cytosols were analyzed for immunoprecipitable protein kinase C, activity, and phorbol ester (PDBu) binding. Results are expressed as means ± SD (n = 3).

DISCUSSION

The down-regulation of immunoprecipitable protein kinase C induced by PMA was studied in ER+ (MCF-7-CG, MCF-7-DF, ZR75-1, and T-47-D) and in ER− (MDA-MB-231, BT-20, and HBL-100) human breast cancer cells. Our affinity-purified antiserum was able to precipitate two protein kinase C forms, a cytosolic 77-kDa form and a membrane-bound 80-kDa form, in tissue and cell extracts of different origin (22, 28). According to our findings, the 80-kDa form represents a highly phosphorylated form of the 77-kDa enzyme (22).

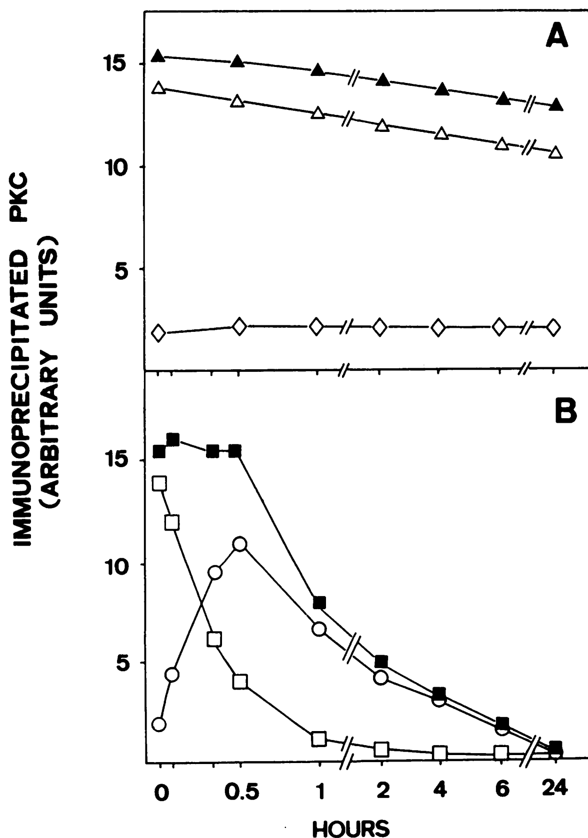

FIG. 3. Effect of PMA on [^{35}S]methionine-prelabeled protein kinase C (PKC). MDA-MB-231 cells were labeled with [^{35}S]methionine for 6 hr and chased for 24 hr in the absence (*A*) and presence (*B*) of 300 nM PMA. Immunoprecipitates were quantified. Protein kinase C of cytosol (△, □), membranes (◇, ○), and total (▲, ■).

Similar results were reported by Huang *et al.* (29), demonstrating a shift in the size of protein kinase C due to autophosphorylation.

Protein kinase C exists as an inactive soluble and an active membrane-bound enzyme (19, 30, 31). Several growth factors induce a rapid and transient membrane association of protein kinase C that reflects its intracellular activation (12–14, 31). Protein kinase C can also be activated by PMA in a time- and dose-dependent, but more permanent, manner (15). Translocation of cytosolic protein kinase C to membranes by PMA is independent of its synthesis, since it can be demonstrated quantitatively on immunoblots (22, 28), by two different [^{35}S]methionine labeling methods, and occurs also in the presence of cycloheximide. There was a quantitative shift of cytosolic protein kinase C to membranes by PMA, although total enzyme activity decreased by 40–60%. This loss may be due either to the extraction procedure or to structural modifications (32, 33). Thereafter, PMA dramatically enhances the down-regulation of the membrane-bound enzyme ($t_{1/2}$, 2 hr) as compared to untreated cells ($t_{1/2}$, >24 hr) (34).

Down-regulation of protein kinase C occurs by a specific protease that preferentially proteolyses the membrane-bound enzyme (35, 36), resulting in a phospholipid- and Ca^{2+}-independent catalytic (50 kDa) and a regulatory phorbol ester-binding unit (30 kDa) (7, 37–39). Our anti-protein kinase C antibody immunoprecipitated only the membrane-bound 50-kDa protein discriminating epitopes on the carboxyl-terminal half of the enzyme (6, 7, 9). The postulated release of the 50-kDa proteolytic fragment from the membrane to the cytosol (40, 41) could not be confirmed by immunoprecipitation due to nonspecific coprecipitation of cytosolic proteins of similar molecular mass, although phospholipid/Ca^{2+}-insensitive kinase activities of proteolytic fragments of protein kinase C were detected in PMA-treated cells (42).

PMA seems not to affect mRNA levels of protein kinase C, suggesting a continuous synthesis of the protein during phorbol ester treatment (43, 44). Membrane-bound 74- and 80-kDa protein kinase C-related proteins were specifically immunoprecipitated after PMA-mediated down-regulation of the active 77- and 80-kDa enzymes. The fact that no significant amounts of this 74/80-kDa doublet could be found in the cytosol indicates its immediate docking into membranes after synthesis. The precise association of the 74/80-kDa doublet to a specific membrane fraction (endoplasmic reticulum, golgi, plasma membrane, etc.) was not defined in the present study. According to the antiserum specificity and V8

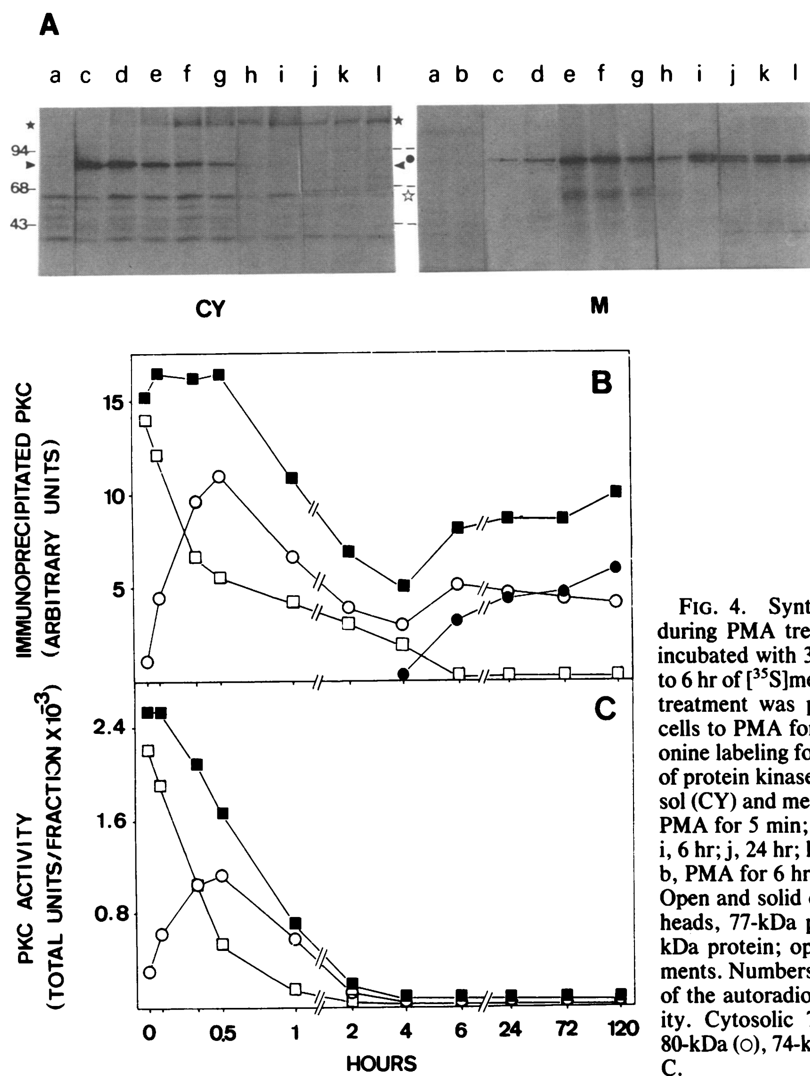

FIG. 4. Synthesis of protein kinase C (PKC) during PMA treatment. MDA-MB-231 cells were incubated with 300 nM PMA during the last 5 min to 6 hr of [^{35}S]methionine labeling. Prolonged PMA treatment was performed by exposing unlabeled cells to PMA for 6–120 hr followed by [^{35}S]methionine labeling for the last 6 hr. (*A*) Autoradiograph of protein kinase C immunoprecipitated from cytosol (CY) and membranes (M). Lanes: c, control; d, PMA for 5 min; e, 30 min; f, 1 hr; g, 2 hr; h, 4 hr; i, 6 hr; j, 24 hr; k, 72 hr; l, 120 hr; a, PMA for 1 hr; b, PMA for 6 hr competed with unlabeled antigen. Open and solid circles, 74/80-kDa doublet; arrowheads, 77-kDa protein kinase C; solid stars, 150-kDa protein; open stars, 50-kDa proteolytic fragments. Numbers on left are kDa. (*B*) Quantification of the autoradiograph. (*C*) Protein kinase C activity. Cytosolic 77-kDa (□), membrane-associated 80-kDa (○), 74-kDa (●), and total (■) protein kinase C.

peptide analysis, both proteins are closely related to functional 77/80-kDa protein kinase C of untreated breast cancer cells despite lack of enzyme activity and phorbol ester binding. It is possible that PMA differentially regulates the expression of protein kinase C genes (45, 46), although it does not affect the amounts of protein kinase C mRNAs (43, 44). Furthermore, PMA may also interfere at posttranslational steps, suggesting that the 74/80-kDa protein doublet represents inactive precursors of the functional protein kinase C forms (77/80 kDa). In this respect, the role of the cytosolic 150-kDa protein that is specifically detected in immunoprecipitates of PMA-treated ER− cells remains to be clarified, since recent findings suggest a covalent dimerization of protein kinase C upon activation of the enzyme (47).

The kinetics of protein kinase C translocation and down-regulation were similar in all human breast cancer cells (42). However, the amounts of the membrane-bound immunoprecipitable 80-kDa protein kinase C-related polypeptide in long-term PMA-treated cells appear to be inversely related to the degree of PMA-induced growth inhibition of the respective cell lines. Resistance to phorbol ester-induced differentiation in promyelocytic leukemia cells correlates with a protein kinase C unable either to translocate (48) or to undergo homologous down-regulation (49). Since removal of

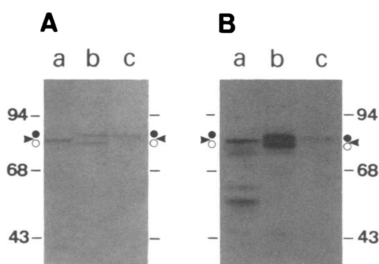

FIG. 5. Immunoprecipitation with antiserum 0442. Autoradiograph of protein kinase C immunoprecipitated with the anti-protein kinase C antibody (*A*) and with antiserum 0442 (*B*). Lane a, control cytosol; lane c, membranes. Lane b, membranes of cells treated for 48 hr with 300 nM PMA. Symbols are the same as in Fig. 4*A*. Numbers are kDa.

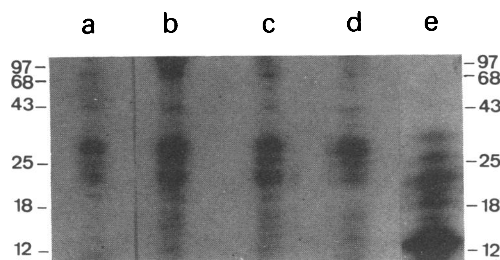

FIG. 6. V8 mapping of protein kinase C and related polypeptides. Autoradiograph of immunoprecipitated protein kinase C bands digested with 0.1 μg of V8 *S. aureus* protease. Lane a, control cytosol (77 kDa); lane b, membranes (80 kDa). PMA long-term-treated membranes. Lane c, 80 kDa; lane d, 74 kDa. Lane e, immunologically unrelated 40-kDa polypeptide. Numbers are kDa.

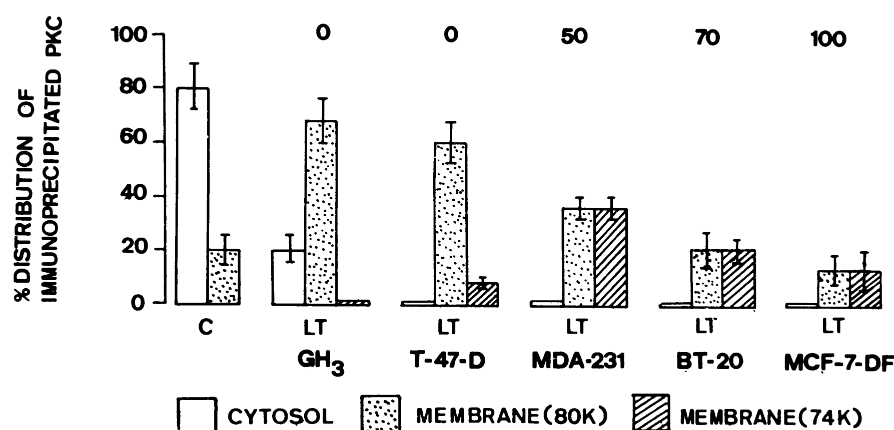

FIG. 7. Synthesis of protein kinase C during prolonged PMA treatment. Immunoprecipitates from cytosols and membranes of rat pituitary GH_3 and human breast cancer cells exposed to 300 nM PMA for 48 hr (LT) as well as of respective control cells (C) were quantified. Total protein kinase C from control cells represents 100%. Results are expressed as means ± SD (n = 3). Numbers above bars indicate percentage growth inhibition by PMA for the respective cell type.

PMA leads to growth resumption of human breast cancer cells concomitant with the reappearance of functional protein kinase C (42), the variable turnovers of the putative protein kinase C precursors may play a role in the different growth response to phorbol esters.

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