Biochemical Pharmacology, Vol. 40, No. 8, pp. 1785–1792, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00
Pergamon Press plc

DISSOCIATION BETWEEN PROTEIN KINASE C CONTENT AND BIOLOGICAL RESPONSIVENESS TO PHORBOL ESTERS IN TUMOR PROMOTER-SENSITIVE (MCF-7) AND RESISTANT (RPh-4) CELLS*

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(*Received* 15 *February* 1990; *accepted* 8 *June* 1990)

Abstract—A cell line (RPh-4) insensitive to the effects of phorbol esters has been isolated from MCF-7 human breast cancer cells. The growth pattern of RPh-4 cells in the presence of 50 ng/mL (80 nM) 12-*O*-tetradecanoylphorbol 13-acetate (TPA) is similar to that of parental MCF-7 cells in the absence of TPA. While phorbol esters inhibit MCF-7 cell proliferation and increase cell volume and protein content, no such effects are observed in RPh-4 cells. TPA affects MCF-7 but not RPh-4 cell cycle in two ways: a G1 block and a delayed passage through G2 phase. Profound alterations in protein kinase C content and activity are observed in RPh-4 versus MCF-7 cells, i.e. (i) a dramatic decline in the cellular enzyme content; (ii) a loss of the capacity to translocate upon acute TPA stimulation for the remainder enzyme; and (iii) a lack of stimulation by phorbol esters of the endogenous M_r 28,000 substrate. However, these striking changes are only transient and rapidly reverse when RPh-4 cells are subcultured in TPA-free medium, with a 60% and an almost total recovery, respectively, after 15 days and 3 months. By contrast, a much lower rate of reversion is observed in terms of cell growth responsiveness to TPA with a total insensitivity to phorbol ester after 80 days and a 50% inhibition of RPh-4 cell proliferation after 3.5 months. Our data clearly demonstrate an apparent dissociation between the cellular protein kinase C content and the biological responsiveness to phorbol ester in the variant RPh-4 cells. Moreover, they suggest that the M_r 28,000 protein phosphorylation event is not directly related to the cell growth arrest induced by phorbol esters in MCF-7 cells.

Tumor promoter phorbol esters such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA¶) induce various biochemical and biological events in cultured cells. They exert striking and often opposite effects at the level of cell growth and differentiation [1, 2]. In the human breast carcinoma MCF-7 cells, TPA and other active phorbol esters inhibit proliferation and cause important changes in cell morphology [3–6]. Such actions are very likely mediated by protein kinase C, shown to be the aporeceptor of these compounds in target cells [7–9]. Indeed, a good correlation is observed between the potency of various phorbol ester derivatives to bind high affinity sites on MCF-7 cells and their efficiency to inhibit proliferation [5]. Furthermore, the permeant diacylglycerol DiC_8, another potent protein kinase C activator, mimics the TPA action on MCF-7 cell growth and morphology [10, 11]. Diacylglycerol and phorbol esters are both capable to rapidly induce the subcellular redistribution of protein kinase C [10] as well as the phosphorylation of a M_r 28,000 endogenous protein [11]. We have recently isolated MCF-7 cell variants (RPh-4) unresponsive to growth inhibition by phorbol esters [12]. We anticipated that such variants could be a useful tool for studying the mechanism of action of TPA. In the present paper, we have compared the protein kinase C activities in the tumor promoter-sensitive (MCF-7) and -resistant (RPH-4) cells. We report an apparent dissociation between the respective enzyme content and cellular responsiveness to phorbol esters in the two cell lines.

MATERIALS AND METHODS

Chemicals. [^{3}H]PDBu (30.8 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). [γ-^{32}P]ATP (0.5–3 Ci/mmol) and [^{32}P]phosphoric acid were from Amersham (Amersham, U.K.). Phorbol diesters and propidium iodide were obtained from the Sigma Chemical Co. (Poole, U.K.). Acrylamide and bisacrylamide were from Biorad (Richmond, CA, U.S.A.). All other chemicals were purchased from Merck (Darmstadt, F.R.G.).

Cell culture. MCF-7 cells were grown at 37° in

* Supported by Institut National de la Santé et de la Recherche Médicale and by Association de la Recherche contre le Cancer.

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¶ The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PDBU, phorbol 12,13-dibutyrate; PDD, phorbol 12,13-didecanoate; PDBz, phorbol 12,13-dibenzoate; DiC_8, 1,2-dioctanoyl-*sn*-glycerol.

RPMI 1640 (Gibco, Uxbridge, U.K.), pH 7.3, supplemented with 2 g/L of sodium bicarbonate, 2 mM glutamine, 1 μM insulin and 0.1 μM transferrin.

Isolation of variant RPh-4 cells from MCF-7 cells. Clone RPh-4 (C4) was isolated from MCF-7 cells by using a microdrop technique [13]. Briefly, 5×10^3 MCF-7 cells/cm^2 adapted to grow in serum-free conditions [14], were treated with 1 μM TPA for 12 days. During the culture, most of the cells detached from culture dishes and died. However, 2 to 3 colonies/10^6 plated cells were still present at the end of this treatment and were then subcultured in the presence of 80 nM (50 ng/mL) TPA. The growth pattern of the obtained cell line (RPh-4) in the presence of the phorbol ester was similar to that of parental MCF-7 cells in the absence of TPA.

Cell growth experiments. MCF-7 and RPh-4 cells were plated at an initial density of 10^5 cells per 35 mm dish in medium containing 0.5% fetal calf serum. This serum concentration was used in order to avoid the formation of cell mass aggregates and to increase the plating efficiency. Twenty-four hours later, cells were placed in serum-free medium and phorbol diesters dissolved in acetone (final concentration of 0.1%) were added. Control cultures received acetone alone. Culture media were changed every 3 days. After 6 days of treatment, cells were dissociated by 0.05% trypsin, 0.02 M EDTA in phosphate-buffered saline, then cell number and volume were measured with a Coulter Counter (Model Z.M.).

Flow cytometric analysis. Cell suspensions (1–2×10^6 cells) were fixed in cold ethanol (70%). Then the cells were treated with RNAse at 37° for 30 min and finally stained with propidium iodide (50 μg/mL in 1% sodium citrate). DNA histograms were obtained by analysing 10^4 cells on an EPICS C flow cytometer (Coulter Electronics).

Subcellular fractionation. Subconfluent cells were rapidly harvested in cold phosphate-buffered saline (PBS), and homogenized in 20 mM Tris–HCl, pH 7.5, containing 0.25 M sucrose, 2 mM EDTA, 2 mM EGTA, 100 μg/mL leupeptin and 5 mM β-mercaptoethanol (buffer A).

The cell lysate was centrifuged for 1 hr at 105,000 *g*. The supernatant was used as the cytosolic fraction. The corresponding pellet was resuspended in buffer A, containing 0.5% NP-40 and briefly sonicated. After incubation at 4° for 45 min it was centrifuged for 1 hr at 105,000 *g*. The supernatant recovered was used as the 0.5% NP-40 extract of the particulate fraction.

DEAE-cellulose chromatography. Cytosol or 0.5% NP-40 extract of particulate fraction obtained from 40×10^6 cells was applied to a DEAE-cellulose column (DE52, 0.8 × 3 cm) equilibrated with 20 mM Tris–HCl, pH 7.5, containing 2 mM EDTA, 1 mM EGTA, 50 μg/mL PMSF and 5 mM β-mercaptoethanol (buffer B). Columns were washed with 10 mL of buffer B, then sequentially eluted in a stepwise manner with 5 mL of buffer B containing 0.13 and 0.35 M NaCl. Protein kinase C and phorbol ester binding activities were immediately assayed, using respectively 40 and 100 μL aliquots. Both activities were recovered in the 0.13 M NaCl fraction for both MCF-7 and RPh-4 cell extracts.

Protein kinase C assay. Protein kinase C was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into H1 histone. The standard assay mixture (200 μL) contained 20 mM Tris–HCl, pH 7.4, 40 μg histone, 10 μM ATP (550 cpm/pmol), 5 mM $MgCl_2$, 40 μL of sample and either 0.5 mM EGTA or 0.5 mM $CaCl_2$, 16 μg of phosphatidyl-serine and 0.6 μg of 1,2-dioleoyl-glycerol. After incubation for 5 min at 30°, 10% trichloroacetic acid was added to stop the reaction. The protein precipitate was dissolved in 1 N NaOH and the incorporation of ^{32}P was measured by scintillation counting in Pico-fluor (Packard).

[^{3}H]*PDBu binding assay.* Phorbol ester binding activity was assayed in a reaction mixture (500 μL) containing 50 mM Tris–HCl, pH 7.5, 0.5 mM $CaCl_2$, 4 mg/mL BSA, 80 μg/mL of phosphatidyl-serine and 10 nM [^{3}H]PDBu. To determine non-specific binding, incubations were made in the presence of 10 μM unlabeled PDBu. Phorbol ester was dissolved in DMSO (final concentration of 0.5%). The incubation was carried out for 2 hr at 2° and terminated by the addition of 4 mL of ice-cold PBS containing 0.04% CHAPS. Bound [^{3}H]PDBu was determined by rapid filtration on polyethylenimine-treated glass fiber GF/B filters. After rapid washing by 8 mL PBS-CHAPS, the filters were placed in scintillation vials. Radioactivity was measured in 10 mL of Pico-fluor (Packard). Non specific binding represented less than 15% of total binding.

Protein phosphorylation. Subconfluent cultures (1×10^6 cells/35 mm dish) were washed twice in a phosphate-free Krebs–Ringer buffer, pH 7.2, containing 20 mM Hepes, 0.1% BSA and 0.2% glucose, and incubated for 2 hr at 37° in 1 mL of the same buffer containing 50 μCi [^{32}P]phosphoric acid. Stimuli were then added for a further 30 min period. Cells were washed twice with cold PBS and 10% trichloroacetic acid (TCA) was added. TCA-precipitated proteins were dissolved in 150 μL of electrophoresis sample buffer containing 0.06 M Tris–HCl, pH 6.7, 2% SDS, 8% glycerol, 2% β-mercaptoethanol and 0.005% Bromophenol blue. Samples were then boiled for 5 min at 90°. Proteins were fractionated by electrophoresis on 4.5 and 12% (w/v) discontinuous SDS–polyacrylamide slab gel. After protein fixation by cold trichloroacetic acid and Coomassie blue staining, the gels were dried, then exposed to Hyperfilm-MP for 48–72 hr.

RESULTS

Effect of phorbol esters on MCF-7 and RPh-4 cell growth

By using a MCF-7 cell line previously adapted to grow in the total absence of serum, we have isolated cell variants (RPh-4) unresponsive to growth inhibition by phorbol esters. The effects of TPA on cell proliferation and volume shown on Fig. 1 were studied after RPh-4 cells were subcultured in a phorbol ester-free medium during two passages (15–18 days). TPA, at all doses that were tested, was unable to inhibit RPh-4 cell proliferation (Fig. 1A) or to modify RPh-4 cell volume (Fig. 1B). In the same conditions, we observed a dose-dependent inhibition of MCF-7 cell proliferation and a concomitant

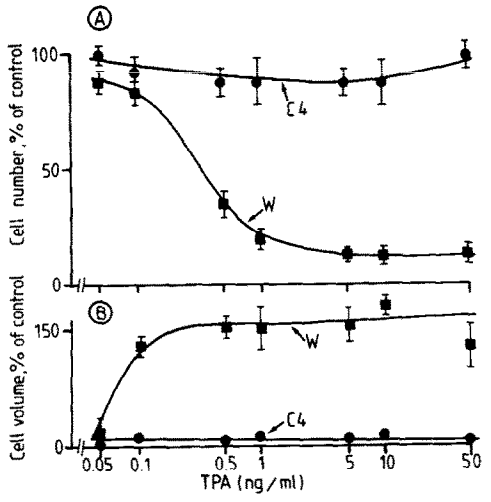

Fig. 1. Effect of increasing concentrations of TPA on cell number (A) and cell volume (B) of MCF-7 (W, ■) and RPh-4 (C4, ●) cells. The measure of cell number and volume was performed after 6 days of treatment. Data are expressed as per cent of control. Each point represents the mean ± SD of 6–9 samples from 2 to 3 separate experiments. In each experiment, cell number and volume were measured on 3 individual dishes.

Table 1. Effect of various phorbol ester derivatives on MCF-7 and RPh-4 cell proliferation

Treatment (6 days)	Cell number (% of control) MCF-7 (W)	RPh-4 (C4)
TPA	17.9 ± 1.4	102.6 ± 5.8
PDBu	29.5 ± 3.6	91.1 ± 1.9
PDD	18.4 ± 1.6	94.3 ± 6.3
PDBz	37.6 ± 6.4	96.7 ± 3.4

Cells were treated as explained in the legend of Fig. 1 but were incubated for 6 days with 5 ng/mL (8 nM) of various phorbol ester derivatives. Results were the mean ± SD of 3 individual measurements and were expressed as per cent of control.

increase in MCF-7 cell volume. The increase in MCF-7 cell volume induced by TPA is accompanied by a two-fold increase in total protein content. On the contrary, no difference in protein content of RPh-4 cells was found after TPA treatment (not shown). The EC_{50} observed for MCF-7 cell growth inhibition was 0.3 ng/mL (0.5 nM), a higher value than the one previously found (0.05 ng/mL, 0.08 nM) when TPA action was studied in the MCF-7 cell line maintained in 5% fetal calf serum [6, 11]. The absence of RPh-4 cell response was not restricted to TPA, as other active phorbol esters like PDBu, PDD and BDBz which inhibit MCF-7 cell proliferation were unable to modify RPh-4 cell growth (Table 1).

Permeant diacylglycerol DiC_8 which has been shown to mimic the phorbol ester effect on MCF-7 cell growth [10, 11] was also ineffective for inhibiting RPh-4 cell proliferation (not shown).

Effect of TPA on MCF-7 and RPh-4 cell cycle

TPA alteration of MCF-7 cell proliferation is associated with specific perturbation of cell cycle events. As shown on Fig. 2. The addition of 5 ng/mL (8 nM) or 50 ng/mL (80 mM) TPA during 96 hr induces a disappearance of cells in the S phase, a slight decrease in the proportion of cells in G2 + M and a corresponding increase of cells in G0-G1. These results agree with those previously reported for MCF-7 cell treated with TPA in the presence of serum [6]. They also corroborate those reported for HeLa cells, where TPA affects cell cycle in two ways, a G1 block and a delayed passage through G2 phase [15, 16]. As expected, in the case of RPh-4 cells, no modification in the proportion of cells in the different phases of the cell cycle was observed after TPA action (Fig. 2).

Protein kinase C content in MCF-7 and RPh-4 cells

As the biological action of phorbol esters are supposed to be the consequence of protein kinase C activation, we investigated the protein kinase C activities in the subcellular fractions of both RPh-4 and MCF-7 cells, by using the *in vitro* histone kinase activity assay.

As shown on Fig. 3, the protein kinase C content in RPh-4 cells depends on the duration of subculture in the absence of TPA. When RPh-4 cells were cultured in phorbol ester-free medium for 8–10 days only, we observed a dramatic loss (approximately 65%) of the protein kinase C activity both in cytosolic and particulate fractions, when compared to the enzyme content in MCF-7 cells. However, when RPh-4 cells were subcultured in the absence of TPA during two passages (15–18 days) protein kinase C activity reached approximately 70% of the values observed in MCF-7 cells. After 13 passages (3 months) in TPA-free medium, RPh-4 cells were found to contain approximately the same amount of protein kinase C than MCF-7 cells.

A similar pattern was observed when [^{3}H]PDBu binding activity was measured in the subcellular fractions of the two cell lines, except that RPh-4 cells cultured in the absence of TPA for 8–10 days were found to contain less than 10% of the PDBu binding activity observed in MCF-7 cells. Only 60% of the binding found in MCF-7 cells was recovered in RPh-4 cells after two passages (15–18 days) in TPA-free medium (not shown).

Effect of TPA on protein kinase C translocation in MCF-7 and RPh-4 cells

Figure 4A illustrates an important feature of the RPh-4 cell behaviour towards an acute TPA treatment. While the phorbol ester induced the rapid subcellular translocation of protein kinase C (probed as the drop in cytosolic enzyme activity) in the parental MCF-7 cell line, no change was observed in RPh-4 cells previously subcultured 8–10 days in the absence of TPA. However, when variant cells were cultured more than 15–18 days in phorbol ester-free medium before the stimulation, the protein kinase C translocation capacity was entirely recovered.

As shown in Fig. 4B, similar pattern was found when [^{3}H]PDBu binding activity was considered

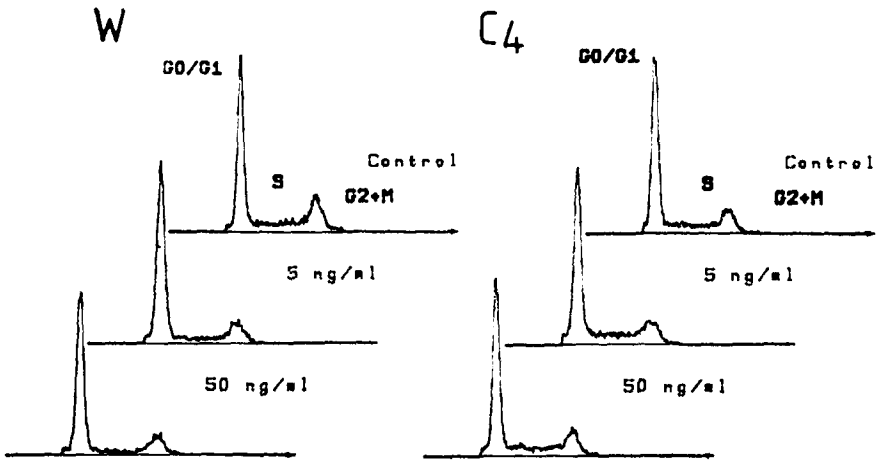

Fig. 2. Effect of TPA on the cell cycle of MCF-7 and RPh-4 cells. Cells (2×10^5) were plated on 35-mm diameter culture dishes. Three days later, 5 ng/mL (8 nM) or 50 ng/mL (80 nM) TPA was added. Ninety-six hours later, cells were harvested and stained with propidium iodide. The per cent of cells (mean ± SE of 3 experiments) in the G0/G1, S and G2/M cell phase were determined from DNA distribution by planimetry: MCF-7: control: G0/G1 = 61.5 ± 2, S = 16.5 ± 1.5, G2/M = 22 ± 2; 5 ng/mL TPA: G0/G1 = 75.5 ± 1.5, S = 10 ± 2, G2/M = 14.5 ± 1.5; 50 ng/mL TPA: G0/G1 = 74 × 1, S = 10 ± 1, G2/M = 16 ± 1. RPh-4: control: G0/G1 = 65 ± 0.5, S = 15 ± 2, G2/M = 20 ± 4; 5 ng/mL TPA: G0/G1 = 66.5 ± 2.5, S = 17 ± 2, G2/M = 16.5 ± 2; 50 ng/mL TPA: G0/G1 = 63.5 ± 0.5, S = 18.5 ± 1, G2/M = 18 ± 1.

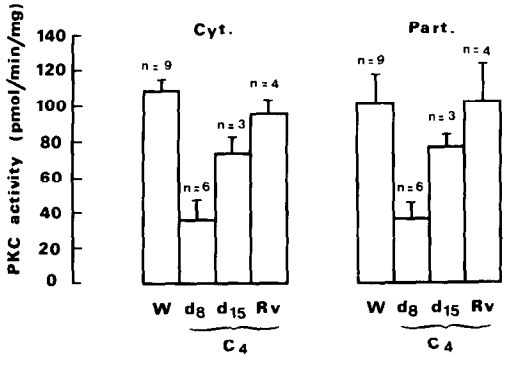

Fig. 3. Protein kinase C content in MCF-7 (W) and RPh-4 (C4) cells. Protein kinase C activities were assayed on cytosolic (Cyt) and particulate (Part) fractions of subconfluent MCF-7 (W) or RPh-4 (C4) cells, following DEAE-cellulose chromatography. RPh-4 cells were subcultured 8–10 days (d8), 15–18 days (d15) or more than 3 months (Rv) in the absence of TPA before the experiment. Data are the means ± SE of 3 to 9 different experiments and are expressed per mg protein loaded on the DEAE-cellulose column.

instead of histone kinase activity, although significant higher values in the extent of translocation were observed by using the phorbol ester binding parameter.

Effect of phorbol esters on protein phosphorylation in MCF-7 and RPh-4 cells

The next intriguing question was to know if the protein kinase C was identically functional in intact RPh-4 and MCF-7 cells. We have previously reported the presence of an endogenous M_r 28,000 specific substrate in MCF-7 cells grown in the presence of serum [11, 17]. Figure 5 shows that TPA as well as PDBu also increase the phosphorylation of the M_r 28,000 protein in MCF-7 cells adapted to grow in the absence of serum. On the contrary, no significant stimulation by the phorbol esters was observed in RPh-4 cells previously subcultured in TPA-free medium for 8–10 days (Fig. 5A). However, a significant labeling of the M_r 28,000 protein was observed in the control cells probably due to small amounts of the highly lipophilic phorbol ester remaining in the cell culture, despite the period of TPA starvation. In any case, the protein kinase C activators were again able to stimulate the M_r 28,000 protein phosphorylation after variant cells were cultured in the absence of TPA during two passages although the extent of the phosphorylation appeared slightly lower than the one observed in parental MCF-7 cells (Fig. 5B).

Effect of long-term culture in TPA-free medium on RPh-4 cell insensitivity to phorbol ester

Since a clear reversion in protein kinase C content and activity was observed in RPh-4 cells as a function of TPA starvation, the stability of these variants in terms of resistance to growth inhibition by phorbol ester could be questioned.

Figure 6 shows that RPh-4 cells remain unresponsive to TPA after 80 days of subculture in TPA-free medium (11 passages). However, after 3.5 months (15 passages) in the absence of phorbol ester, the variant cells became sensitive to TPA in a dose-dependent manner, although maximal inhibition reached only 50% instead of 75% in the case of parental MCF-7 cells.

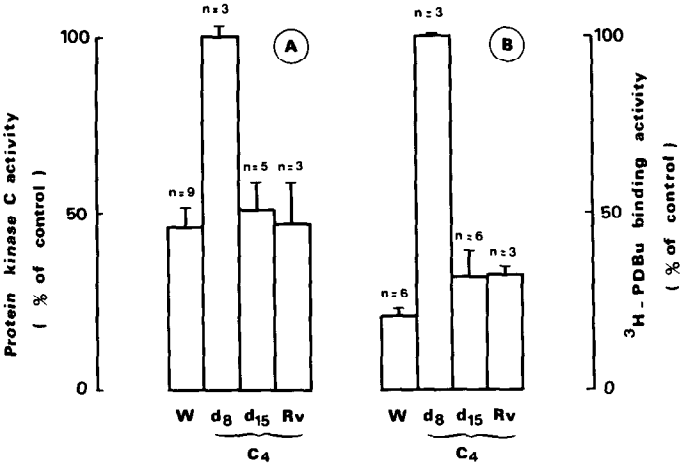

Fig. 4. Effect of acute cell stimulation by TPA on protein kinase C and [^{3}H]PDBu activities. Subconfluent MCF-7 (W) or RPh-4 (C4) cells were incubated for 10 min in the absence (control) or in the presence of 100 ng/mL TPA. RPh-4 cells were subcultured in TPA-free medium before the experiment as indicated in the legend of Fig. 3. Protein kinase C (A) and [^{3}H]PDBu binding (B) activities were measured in cytosolic fractions following DEAE-cellulose chromatography. Results are expressed as per cent of the respective control and are the means ± SE of 3 to 9 experiments.

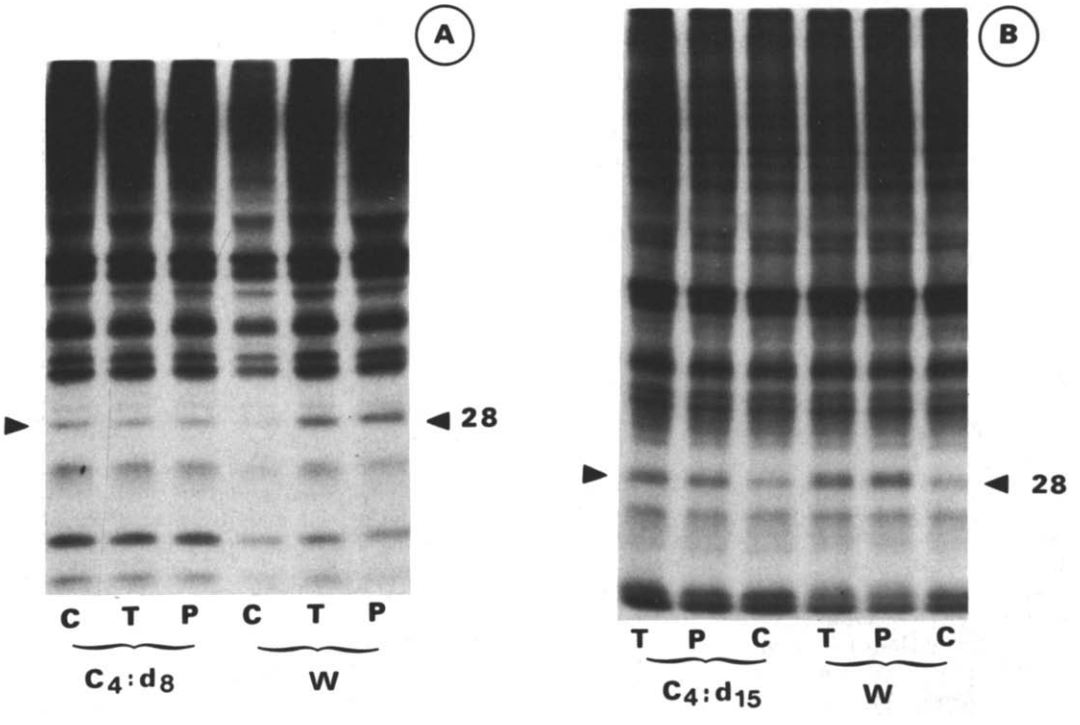

Fig. 5. Effect of TPA and PDBu on the protein phosphorylation pattern in intact MCF-7 and RPh-4 cells. Subconfluent MCF-7 (W) and RPh-4 (C4) cells were incubated for 30 min in phosphate-free Krebs–Ringer buffer containing 50 μCi [^{32}P]phosphoric acid in the absence (C) or in the presence of 100 ng/mL TPA (T) or 100 ng/mL PDBu (P). TCA-precipitated proteins were analysed by SDS–polyacrylamide gel electrophoresis and autoradiography. The arrow indicates the position of the M_r 28,000 protein. RPh-4 cells were subcultured in TPA-free medium for 8 days (A) or 15 days (B) before the experiment.

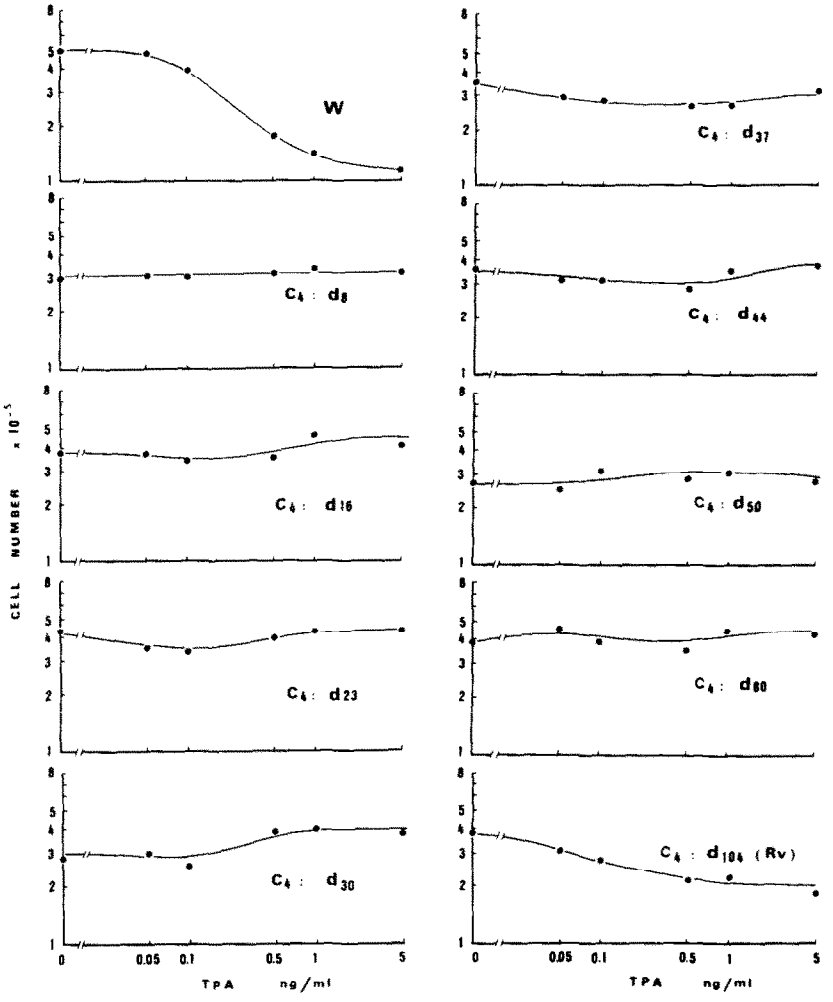

Fig. 6. Effect of long-term culture in TPA-free medium on RPh-4 cell insensitivity to phorbol ester. MCF-7 (W) and RPh-4 (C4) were plated at an initial density of 0.8 to 1 × 10^5 cells per 35-mm dish and counted after 6 days of culture in the absence or presence of increasing concentrations of TPA. RPh-4 cells were subcultured in TPA-free medium for 8 (d8), 16 (d16), 23 (d23), 30 (d30), 37 (d37), 44 (d44), 50 (d50), 80 (d80), or 104 (d104, Rv) days before the experiment. Each point represents the mean of three individual measurements with maximum variation lower than 10%.

DISCUSSION

We show here that phorbol ester TPA induces in MCF-7 cells adapted to grow in the absence of serum, the same events as those previously reported for MCF-7 cells maintained in the presence of serum [17–19], namely (i) subcellular translocation of protein kinase C (measured in term of [^{3}H]PDBu binding as well as Ca^{2+}/phospholipid-dependent histone kinase activities); (ii) phosphorylation of the endogenous M_r 28,000 substrate upon stimulation of intact cells; and (iii) striking inhibition of cell proliferation concomitant to changes in cellular morphology.

RPh-4 cell variants unresponsive to growth inhibition by TPA were obtained by using the serum-free MCF-7 cell line and were routinely cultured in the presence of 50 ng/mL (80 nM) TPA. We show in this paper profound alterations in protein kinase C content and activity of RPh-4 variants versus MCF-7 cells, namely (i) dramatic decline in protein kinase C content; (ii) loss of capacity to translocate upon TPA stimulation for the remainder enzyme; and (iii) lack of stimulation by phorbol esters of the M_r 28,000 protein phosphorylation.

Such acute modifications in the protein kinase C behaviour have been reported in HL-60 variants unresponsive to TPA in terms of cell differentiation, with impairment of enzyme translocation [20] or quantitative alteration of specific protein phosphorylations [21, 22]. This latter parameter is also affected in tumor promoter-resistant pre-neoplastic JB6 cells [23]. Also, recent report indicates that the protein kinase C content is markedly decreased in cell homogenates of the phorbol ester-resistant subline KG1-a obtained from human acute myeloid leukemia KG1 cells [24]. By contrast, no change in the [^{3}H]PDBu binding on intact cells was observed in these KG1-a variants when compared to parental cells [25]. Also, the resistance to phorbol ester-induced differentiation in different HL-60 variants

was not associated with significant change in the affinity or number of [^{3}H]PDBu binding sites when measured on intact cells [26–29].

In our study, the striking changes in protein kinase C content and activity were observed in RPh-4 cells subcultured in the absence of TPA for a short period of time (8–10 days) but rapidly reverse as a function of the duration of TPA starvation, with an almost total recovery observed after 3 months for protein kinase C content and activity (subcellular translocation and protein phosphorylation of the M_r 28,000 protein substrate). By contrast, a much lower rate of reversion was observed in terms of cell growth responsiveness to TPA. RPh-4 cells were totally resistant to TPA after 80 days of subculture in phorbol ester-free medium and were only partially sensitive to TPA after 3.5 months (inhibition of cell growth by 50% in RPh-4 cells versus 75% in MCF-7 cells). In other words, our data clearly demonstrate an apparent dissociation between the cellular protein kinase C content and the biological cell response. By contrast, in phorbol ester-resistant HL-60 variants R1B6, where [^{3}H]PDBu binding was markedly decreased when compared to parental cells, the reversion of the variants to sensitivity to phorbol esters has been shown to be closely correlated to the up-regulation of the cytosol and membrane phorbol ester receptor [30].

The transient decline in protein kinase C content and activity that we observed in RPh-4 cells may be simply the consequence of the down-regulation of the enzyme induced upon long-term treatment with TPA [18, 19]. However, we have previously shown that the low percentage of protein kinase C activity remaining after long exposure of MCF-7 cells to TPA was able to produce a maximal phosphorylation of the M_r 28,000 specific substrate [11]. By contrast, in RPh-4 cells, the extent of phosphorylation of the M_r 28,000 protein apparently depends on the protein kinase C cellular content. Moreover, control experiments using [^{3}H]TPA showed that RPh-4 cells were virtually TPA-free after 15 days of culture in phorbol ester-free medium.

The major finding of the present report concerns the relationship between the phosphorylation of the M_r 28,000 protein and the responsiveness to TPA in terms of cell growth inhibition: the data observed with RPh-4 cells clearly demonstrate that this specific protein phosphorylation is not directly related to the inhibition of cell proliferation, as the M_r 28,000 protein could be phosphorylated upon phorbol ester stimulation in RPh-4 cells totally unresponsive to TPA for growth inhibition. As a consequence, it can be postulated that the phosphorylation of the M_r 28,000 protein may be necessary but not sufficient to induce growth arrest in MCF-7 cells. The biochemical lesion induced by TPA in MCF-7 cells could take place at a step distal to the M_r 28,000 protein phosphorylation event.

Other protein phosphorylations, that we do not observe by using mono-dimensional electrophoresis, could be more directly implicated in the biological cell response. Such discrete phosphorylations could occur at the nuclear level in MCF-7 cells and not in RPH-4 cells. Whether the two cell lines possess different protein kinase C isoforms with distinct substrate specificity remains to be investigated. The existence of such protein kinase C subspecies is now well documented [31–33]. Also, recent reports demonstrate that TPA could induce the translocation of protein kinase C in a directional way depending on the cell type. In HL-60 cells where the phorbol ester causes an inhibition of cell growth, the translocation occurs from the cytosol to the plasma membrane [34] while in 3T3 fibroblasts, where TPA induces cell proliferation, the enzyme is partly redistributed from the cytosol to the nuclear fraction [35]. Whether such bi-directional translocation of protein kinase C may be involved during MCF-7 and RPh-4 cells treatment with phorbol esters has to be tested.

Finally it may be objected that TPA could exert its action by a mechanism (other receptor?) other than the activation of protein kinase C. However, our previous results do not favor such an hypothesis. Indeed, all the enzyme activators tested i.e. phorbol esters, permeant diacylglycerol DiC_8 and phospholipase C induced MCF-7 cell growth arrest [5, 11]. The differences observed in the present study between the rates of reversion of protein kinase C content/activity, on one hand, and of RPh-4 cell growth inhibition, on the other hand, could rather suggest subtile regulation by the enzyme of molecular events involved in biological cell response. In any case, the M_r 28,000 protein phosphorylation does not appear anymore as an unambiguous index of cell proliferation inhibition of the MCF-7 cell line, although this specific endogenous substrate always appears as an excellent marker of protein kinase C functionality.

Acknowledgements—The authors gratefully thank Mrs Simone Clamens for her technical assistance and Mrs Monique Larribe for typing the manuscript. We are indebted to Dr F. Roubinet for his precious collaboration in flow cytometry studies.

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