Comparison of Effects of Bryostatins 1 and 2 and 12-O-Tetradecanoylphorbol-13-acetate on Protein Kinase C Activity in A549 Human Lung Carcinoma Cells

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ABSTRACT

Activators of protein kinase C (PKC), such as 12-O-tetradecanoylphorbol-13-acetate (TPA) and bryostatins 1 and 2, inhibit the growth of A549 cells. At high concentrations the bryostatins do not affect cell growth. Here the hypothesis has been tested that modulation of A549 cell growth is the consequence of agent-induced changes in location or extent of cellular PKC activity. PKC activity was measured after semi-purification with nondenaturing polyacrylamide gel electrophoresis in the cytosol and the particulate fraction of A549 cells. When cells were exposed to TPA or mezerein, PKC activity underwent rapid and concentration-dependent translocation from the cytosol to the membrane. TPA at 0.1 μM or mezerein at 1 μM caused almost complete translocation within 30 min. Incubation with bryostatins 1 or 2 also led to enzyme translocation, which was, however, much weaker than that observed with the tumor promoters. Neither 4α-phorbolidecanoate nor the synthetic diacylglycerols 1,2-sn-dioctanoylglycerol or l-oleoyl-2-acetyl-sn-glycerol mimicked TPA in this way. Exposure of cells to TPA or the bryostatins for longer than 30 min caused the gradual disappearance of total cellular PKC activity. PKC downregulation was concentration dependent and complete after 24 h. A549 cells which had acquired temporary resistance toward the growth-arresting potential of TPA were completely devoid of any measurable PKC activity. The bryostatins were potent inhibitors of the binding of [3H]phorbol-12,13-dibutyrate to its receptors in intact cells, and the inhibition was dependent on bryostatin concentration. The results support the contention that PKC is involved in the mediation of growth inhibition caused by TPA or the bryostatins. However, the relationship between growth arrest and PKC translocation or downregulation seems to be a complex one.

INTRODUCTION

Tumor-promoting phorbol esters such as TPA elicit a large variety of responses in cultured cells, including stimulatory or inhibitory effects on growth and differentiation (1–3). In A549 human lung carcinoma cells, nontoxic concentrations of TPA induce growth arrest accompanied by a change in cell morphology (4). The biological effects of TPA are thought to be mediated, at least in part, by PKC (5, 6), a pivotal enzyme in the transmembrane signalling system involving the receptor-coupled breakdown of inositol phospholipids. The bryostatins, macrocyclic lactones isolated from marine bryozoans (7, 8), are, like TPA, activators of PKC (9, 10). The bryostatins are particularly interesting compounds from a chemotherapeutic point of view because they possess antineoplastic activity against the murine P388 lymphocytic leukemia (11). A puzzling feature of their biological activity is the fact that, in many cell lines, they are not only agonistic with TPA but also able to antagonize biochemical responses elicited by themselves or by TPA (12–15). We have recently shown that, in A549 cells, bryostatins 1 and 2 inhibit growth maximally at 10 nM and 0.1 μM, respectively, but at higher concentrations, they block both their own inhibitory effect and the antiproliferative action of phorbol esters or of mezerein, another diterpenoid tumor promotor (16).

In many cell types, activators of PKC, such as TPA, induce a rapid translocation of PKC from the cytosol to the membrane, followed by a progressive disappearance of enzyme activity (17). In the work described here, an attempt has been made to correlate the growth-modulatory activity of TPA and bryostatins with their ability to modulate PKC activity. In particular, the hypothesis has been tested that the change in the growth pattern of A549 cells caused by TPA and the bryostatins is the result of their ability to elicit enzyme translocation or down-regulation. As part of the investigation PKC activity was measured in cells which have been desensitized to the growth-inhibitory action of TPA (4).

MATERIALS AND METHODS

Chemicals. TPA, PDBu, 4α-PDD, OAG, DÌC8, mezerein, and biochemicals for the enzyme assays were purchased from Sigma (Poole, United Kingdom). Bryostatins 1 and 2 were isolated as described previously (7, 8). Tissue culture reagents and media were obtained from Gibco (Paisley, United Kingdom), and [3H]PDBu and [γ-32P]ATP were from the New England Nuclear Division of Dupont Europe (Stevenage, United Kingdom). Stock solutions of tumor promoters, bryostatins, and diacylglycerols were prepared in DMSO and stored at −20°C. The final concentration of DMSO in the culture medium did not exceed 0.5%. DMSO was added to control cultures, and at this concentration, it did not affect growth or PKC activity.

Cell Culture Conditions. A549 lung carcinoma cells were obtained from the American Type Culture Collection and routinely cultured in nutrient (Ham's F-12 medium supplemented with 10% fetal calf serum) as described previously (4). For the PKC assays, cells were grown in Petri dishes (14-cm diameter) and used when they were 60 to 90% confluent.

Preparation of Cytosol and Membrane Fraction. Fractions were separated essentially as described by Regazzi et al. (18). Briefly, cells were scraped off the dish and suspended in homogenization buffer (20 mM Tris; 2 mM EGTA; 2 mM EDTA; 2 μg/ml of aprotinin; 20 μg/ml of leupeptin; 6 mM mercaptoethanol, pH 7.4) before disruption by sonication. Cytosolic supernatant (designated the "cytosolic fraction") and membrane pellet were obtained by centrifugation (100,000 × g) at 4°C for 30 min. The membrane pellet was resuspended in homogenization buffer containing 1% (w/v) Nonidet P-40 before renewed sonication and centrifugation. The resulting supernatant (designated the "particulate" or "membrane fraction") or the cytosolic fraction was supplemented with 15% (w/v) glycerol and stored for up to 24 h at −20°C.

Polyacrylamide Gel Electrophoresis. PKC in the fractions was partially purified by nondenaturing polyacrylamide gel electrophoresis according to Fabbro et al. (19) using a Bio-Rad Model 175 tube gel cell apparatus. Conditions for the gel electrophoresis were as follows. Temperature was maintained at 4°C. Voltage was at 100 V while the dye (Amaranth) boundary moved through the stacking gel (running time, approximately 4 h) and at 160 to 180 V while the boundary moved through the resolving gel (total running time, 7 to 9 h). After completion of the electrophoresis, the stacking gel was discarded, and...
the resolving gel was placed in a polystyrene tube and frozen on top of dry ice. Gels were sliced into 1-mm sections using a Mickle gel slicer. The gel discs were transferred to tubes containing elution buffer, and the enzyme was eluted from slices overnight by gentle shaking at 4°C.

Protein Kinase C Assay. PKC activity was assayed in gel eluates using a microassay technique adapted from the method described by Kikkawa et al. (20) using protamine sulfate as substrate. Aliquots of the gel eluate (150 µl) were pipetted into the wells of a 96-well microtiter plate and allowed to equilibrate at 32°C. The enzyme reaction was initiated by the addition of protamine assay mixture (50 µl) using a multichannel pipette. Final concentrations or amounts of assay components in the incubate (200 µl) were: 20 mM Tris-HCl (pH 7.4); 10 mM Mg(NO3)2; 24 µM ATP; 0.5 µCi of [γ-32P]ATP; and 100 µg of protamine sulfate. After a 10-min incubation, aliquots (150 µl), seven at a time, were removed with the multichannel pipette and immediately spotted onto rectangles of Whatman No. 17 filter paper (approximately 2 cm x 6 mm) arranged in a multiwell microtiter plate. The reaction was quenched by immersion in ice-cold 10% (w/v) trichloroacetic acid. Filter paper strips were washed for 1 h in 10% (w/v) trichloroacetic acid, the washing solution being changed every 15 min. Paper strips were briefly soaked in methanol and dried by heat. Radioactivity was counted in 5 ml of Lumac gel scintillator using a Packard Tricarb CA2000 scintillation counter. One unit of PKC activity is defined as the amount of enzyme which incorporated 1 pmol of phosphate from [γ-32P]ATP into protamine sulfate per min at 32°C.

Phorbol Ester Receptor Binding Studies. The conditions of the assay, in which the ability of the bryostatins to compete with the binding of [3H]PDBu to intact A549 cells was measured, were as described before (4). The concentration of [3H]PDBu was 50 nM (specific activity, 10.2 Ci/mmol) which was complemented with 50 µM unlabeled PDBu. Aliquots of the gel eluate (150 µl) were pipetted into the wells of a 96-well microtiter plate and allowed to equilibrate at 32°C. The enzyme reaction was initiated by the addition of protamine assay mixture (50 µl) using a multichannel pipette. Final concentrations or amounts of assay components in the incubate (200 µl) were: 20 mM Tris-HCl (pH 7.4); 10 mM Mg(NO3)2; 24 µM ATP; 0.5 µCi of [γ-32P]ATP; and 100 µg of protamine sulfate. After a 10-min incubation, aliquots (150 µl), seven at a time, were removed with the multichannel pipette and immediately spotted onto rectangles of Whatman No. 17 filter paper (approximately 2 cm x 6 mm) arranged in a multiwell microtiter plate. The reaction was quenched by immersion in ice-cold 10% (w/v) trichloroacetic acid. Filter paper strips were washed for 1 h in 10% (w/v) trichloroacetic acid, the washing solution being changed every 15 min. Paper strips were briefly soaked in methanol and dried by heat. Radioactivity was counted in 5 ml of Lumac gel scintillator using a Packard Tricarb CA2000 scintillation counter. One unit of PKC activity is defined as the amount of enzyme which incorporated 1 pmol of phosphate from [γ-32P]ATP into protamine sulfate per min at 32°C.

Translocation of PKC Activity. When A549 cells were incubated with the tumor promoters TPA or mezeerin for 30 min, enzyme activity was translocated in a dose-dependent fashion from the cytosol to the particulate fraction (Fig. 1). In contrast, neither 4α-PDD, which is inactive as a tumor promoter (1), nor DiC8 or OAG, synthetic analogues of naturally occurring diacylglycerols, caused significant translocation of PKC. DiC8 was able to elicit a slight decrease in cytosolic PKC activity by 8% at the highest concentration used (0.3 mM) (results not shown). At this concentration DiC8 was cytotoxic. Exposure of cells to bryostatin 1 or 2 resulted in partial translocation of enzyme activity from the cytosol to the membrane (Fig. 2). The relative potency of tumor promoters and bryostatins in inducing PKC translocation to the membrane was TPA > mezeerin > bryostatin 1 = bryostatin 2.

Downregulation of PKC Activity. Phorbol esters are known to modulate not only the subcellular distribution of PKC activity, but also the total amount of PKC activity in cells (17). In order to test the hypothesis that TPA and the bryostatins cause downregulation of PKC in A549 cells, enzyme activity was measured in cells which had been incubated with TPA or the bryostatins for up to 24 h. TPA elicited the disappearance of enzyme activity in a concentration-dependent manner (Fig. 3). On exposure to 10 nM TPA, a concentration which causes maximal growth inhibition in these cells (4), downregulation was observed after incubation for 5 h (Fig. 3a), whereas at 0.3 µM TPA, enzyme activity had almost totally disappeared already after 1 h of incubation (Fig. 3c). Likewise, incubation of cells with bryostatin 1 (Fig. 4) or bryostatin 2 (results not shown) led to enzyme downregulation. As in the case of TPA, there was a marked concentration dependency in the pattern of enzyme disappearance. The rate at which PKC was downregulated in the presence of 1 µM bryostatin 1 (Fig. 4b), a concentration at which it abolishes its own growth-arresting activity (16), was considerably faster than that observed with bryostatin 1 at 10 nM, the maximal growth-inhibitory concentration (Fig. 4a).

Previously we reported that A549 cells acquire reversible resistance towards the growth-inhibiting properties of TPA under two conditions (4). When incubated with TPA for more than 5 to 6 days, cells became desensitized unless they were detached and subcultured, upon which they regained sensitivity. After subculturing cells in the continued presence of TPA for 9 wk, more permanent desensitization was achieved. Cells treated with TPA to acquire either of the described phenotypes of resistance towards the growth-arresting potential of TPA were completely without any measurable PKC activity, whereas naive cells contained 1375 ± 103 units of PKC/mg of protein in the cytosol and 106 ± 36 units/mg of protein in the particulate fraction (mean ± SD, n = 17).

Binding of the Bryostatins to the Phorbol Receptor. Bryostatins possess high affinity for the phorbol ester receptor (9). The hypothesis was tested that their ability to inhibit growth at low concentrations or to abolish their growth inhibition at higher concentrations in A549 cells is related to a concentration-dependent change in their pattern of binding to the phorbol ester receptor. To that end, the ability of bryostatins 1 and 2 to inhibit the binding of [3H]PDBu to its receptor in intact A549 cells was studied. Fig. 5 shows that both bryostatins are potent inhibitors of PDBu binding and that this effect is dependent on bryostatin concentration. At 1 µM, the concentration at which they abolish their own growth inhibition, the ability of the
The relationship between modulation of PKC activity caused by tumor promotor such as TPA or mezerein and their effect on cell proliferation and differentiation is unclear. This issue by tumor promotors such as TPA or mezerein and their effect on cellular PKC activity, because their ability at high concentrations to abolish growth inhibition was not paralleled by decreases in the rate of enzyme activity. The fact that TPA, mezerein, and the bryostatins abolish their own growth-inhibitory activity at concentrations which exceed their maximal growth-arresting concentrations by a factor of 10 (16). In view of this difference in effect on cell growth between the bryostatins and TPA, it is noteworthy that enzyme translocation caused by the bryostatins was weaker than that elicited by TPA. However, the concentration dependency of the rate at which PKC was downregulated by both agents was similar.

The following three observations render it unlikely that the effects of the tumor promoters and bryostatins on growth are a direct consequence of the observed changes in PKC activity.

(a) The concentrations of agents required to cause detectable enzyme translocation were 10- to 100-fold higher than those necessary to arrest cell growth.

(b) The time course of TPA-induced disappearance of total cellular PKC activity demonstrated here reflects the time course of appearance of growth inhibition (16). However, cells which had regained their growth potential after 5 days of incubation in the presence of TPA or cells which had acquired resistance to TPA-induced growth inhibition by subculturing them for 3 mo with TPA had not regained any measurable PKC activity. This result contrasts with observations using an HL-60-derived cell type made resistant towards the differentiation-inducing action of TPA (25). In these resistant cells, the extent of PKC activity was similar to that found in the parent cell line, but PKC was not translocated on exposure to TPA. We showed previously that the binding of PDBu to the phorbol ester receptor in intact desensitized A549 cells is 75% of control cells after exposure to PDBu for 5 days and 77% in the more permanently desensitized cells (4). The results obtained here suggest that the receptor population in the desensitized cells is either not functional PKC or a subspecies of the enzyme which does not phosphorylate protamine under the conditions of the assay.

(c) The third finding presented here which casts doubt on a direct causal relationship between effect on growth of A549 cells and alterations in PKC activity is concerned with the bryostatins. The paradoxical nature of the response of cell growth to different concentrations of the bryostatins was not reflected by a concentration-dependent reversal in their effects on cellular PKC activity, because their ability at high concentrations to abolish growth inhibition was not paralleled by inhibition of PKC downregulation and consequent maintenance of enzyme activity. The fact that TPA, mezerein, and the bryostatins cause PKC translocation and downregulation and induce growth inhibition is consistent with a role for PKC in...
growth arrest. However, the nature of the relationship between growth potential and PKC activity seems to be complex. Furthermore, PKC does not appear to be directly involved in the mediation of the phorbol ester antagonistic effect of the bryostatins at high concentrations.

The inability to explain the inhibition of the growth of A549 cells as the direct consequence of changes in PKC location or PKC activity is in accordance with conclusions drawn from experiments with phorbol esters in other cell lines. For example, neither activation of PKC and the phosphorylation of specific substrates nor the loss of total PKC activity was considered to be directly responsible for the ability of TPA to induce differentiation in HL-60 cells (23). Instead, other specific changes in cellular metabolism which resulted in a decrease in c-myc RNA levels seemed to be involved.

Now it is clear that PKC is not one enzymatic entity but a family of 7 or more related proteins (26). The tissue- and cell-specific expression of the different PKC subspecies suggests that each member of the enzyme family might have a different function in the mediation of cellular responses to external stimuli. Recently it has been shown that, on treatment with TPA, the different PKC subspecies coexpressed in KM3 cells are downregulated at different rates (27). It remains to be investigated whether the modulation of the activity of a specific, perhaps minor, PKC isoform in A549 cells parallels the growth-modulatory action of the tumor promoters and bryostatins more closely than do the changes in total PKC activity described here.

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