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PHORBOL ESTERS INHIBIT THE PROLIFERATION OF MCF-7 CELLS

POSSIBLE IMPLICATION OF PROTEIN KINASE C*

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Abstract—The effect of tumor promoter phorbol esters on cell proliferation was investigated in human breast cancer cell line MCF-7. During a 4-day culture period, the various phorbol ester derivatives TPA, PDD, PDBu, PDBz and PDA inhibited the proliferation of MCF-7 cells in a dose-dependent manner, with respective IC_{50} of 0.06, 0.75, 2.4, 3.6 and 15 × 10^{-9} M. The 4-*O*-met-TPA, αPDD and αPHR were ineffective at 2 × 10^{-7} M, the highest concentration tested. Using a ^{3}H-PDBu probe, we demonstrated the presence of specific, high affinity binding sites in intact cultured cells, with a K_d of about 9 × 10^{-9} M. Unlabelled TPA, PDD, PDBU and PDBz competed with ^{3}H-PDBu with respective IC_{50} of 35, 12.5, 150 and 220 × 10^{-9} M. High concentrations of PDA, 4-*O*-met-TPA and αPDD slightly inhibited the ^{3}H PDBu binding, whereas αPHR did not until 10^{-5} M.

The correlation that we observed between the relative potencies of the various phorbol derivatives for inhibiting both PDBu binding and cell proliferation, suggests that tumor promoter phorbol esters may induce growth arrest in MCF-7 cells by the mediation of protein kinase C.

Tumor promoters are compounds which, although not carcinogenic by themselves, can induce skin tumors in animals that have repeatedly received a subthreshold dose of chemical carcinogen [1, 2]. The croton oil diterpene phorbol esters are the most potent of these co-carcinogenic compounds, and the most efficient of them is the 12-*O*-tetradecanoyl-13-acetate, TPA [3].‡ Phorbol esters have been shown to produce various and often opposite biological responses in different types of cultured cells. TPA can promote the transformation of fibroblasts *in vitro*, subsequent to initiation by carcinogens, and further enhances the transformed properties of virus-infected fibroblasts [4, 5]. In many cell systems, such as pre-adipocytes 3T3 cells [6], mouse mammary epithelial cells [7], ovine thyroid cells [8], or avian myoblasts [9], TPA stimulates cell proliferation and inhibits cell differentiation. In others, such as human leukemia cells HL 60 [10], or murine pre-B lymphocytes [11], it induces growth arrest and differentiation. In the human breast cancer cell line MCF-7, high concentrations of TPA have been reported to produce such an inhibition of cell proliferation [12]. In the various cell types studied, TPA exerts a large variety of biochemical effects, including a rapid stimulation of ion fluxes, an increase in intracellular pH, a decrease in the affinity of the surface receptors for EGF, an induction of gene transcription, a modulation of phospholipid metabolism, or an induction of protein phosphorylation [13, 14]. In the MCF-7 cells, it has been reported [12] that TPA could inhibit EGF binding as in other cell systems such as human epidermal carcinoma cells A 431 [15], mouse 3T3 cells [16], or HeLa cells [17]. However, the precise mechanism of action of TPA remains unknown. Calcium- and phospholipid-dependent protein kinase (protein kinase C), which is widely distributed in many species and tissues, has been shown to be directly activated by TPA, in rat brain *in vitro* [18] and, in human platelets *in vivo* [18]. The tissue distribution of the phorbol ester binding sites is apparently similar to that of protein kinase C, and identical amounts are observed in rat brain [19, 20]. Moreover, protein kinase C from mouse brain co-purifies with the phorbol dibutyrate binding activity and has been recently proposed as the possible apo-receptor of TPA [21]. Therefore, in MCF-7 cells, the question arises to know whether TPA could exert its inhibitory action on cell proliferation by the mediation of protein kinase C. In this paper, we attempt to demonstrate the existence of saturable and highly specific binding sites for phorbol esters in MCF-7 cells. The second goal of this paper is to produce pharmacological evidence that the phorbol ester binding sites detected with ^{3}H-PDBu on cultured intact MCF-7 cells mediate the biological response of these cells, namely growth arrest, to the tumor promoter phorbol esters. We attempt to correlate the relative potencies of different phorbol esters to inhibit cell proliferation and ^{3}H-PDBu binding on intact cells.

* Preliminary results have already been presented at the 5th French Congress of Endocrinology, October 1985 (M. Issandou, S. Jozan, F. Bayard and J. M. Darbon, *Annales d'Endocrinologie*, **46**, Abstract 108 (1985)).

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‡ Abbreviations used: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PDBu, phorbol 12,13-dibutyrate; PDD, phorbol 12,13-didecanoate; PDBz, phorbol 12,13 dibenzoate; PDA, phorbol 12,13 diacetate; 4-*O*-met-TPA, 4-*O*-methyl-TPA; αPDD, 4α-phorbol 12,13-didecanoate; αPHR, 4α-phorbol; DMSO, dimethyl sulfoxide.

MATERIALS AND METHODS

Materials. ^{3}H-PDBu (specific activity 30.8 Ci/mmol) was purchased from New England Nuclear. Non-radioactive phorbol esters were obtained from Sigma. All other chemicals were from Merck.

Cell cultures. Gift of M. Rich (Michigan Cancer Foundation, Detroit), MCF-7 cells were grown in RPMI 1640 (Gibco), pH 7.3 at 37°, supplemented with 2 g of sodium bicarbonate per litre, 2 mM glutamine, 1 μM insulin (Novo Laboratories) and 5% foetal calf serum (Seromed). Cells were incubated in 35 mm diameter dishes (Nunc, Roskilde, Denmark) at 37° in humidified 5% CO_2/95% air. Culture media were changed every two or three days.

^{3}H-PDBu binding assay. Subconfluent cultures (1 million of cells/dish) were washed twice with RPMI containing 0.1% bovine serum albumin (BSA) and incubated 30 min at 37° in 1 ml of the same fresh medium with 5–100 nM ^{3}H-PDBu. To determine nonspecific binding, incubations were made in the presence of 10 μM unlabelled PDBu. Phorbol derivatives were dissolved in DMSO (final concentration of 0.5%). At the end of incubation, cells were rapidly washed three times with 2 ml of ice-cold RPMI containing 0·1% BSA, then solubilized in 1 ml of 1 N NaOH. The solubilizing solution was assayed for radioactivity in 10 ml of Pico-Fluor 15 (Packard).

Cell growth measurement. MCF-7 were plated at an initial density of 1 to 1.5 × 10^5 cells per 35 mm dish and were maintained in RPMI medium, supplemented with 5% foetal calf serum (FCS). After 24 hr the medium was replaced by fresh RPMI, 5% FCS containing different concentrations of the various phorbol ester derivatives which were dissolved in acetone. Control dishes received the same volume of acetone (final concentration of 0.1%). After 4 days of culture, cells were dissociated by 0.05% trypsin (Gibco), 0.02 M EDTA in phosphate-buffered saline and counted with a Coulter counter.

RESULTS

^{3}H-PDBu binding on MCF-7 cells

Figure 1A illustrates the concentration dependency of the ^{3}H-PDBu binding on intact cultured MCF-7 cells at 37°. The specific binding saturated between 35 and 67 nM. Scatchard analysis of the binding data indicates a single class of binding sites with an approximate K_d of 9 nM and a number of binding sites of about 360,000 sites/cell (Fig. 1B).

Effects of various phorbol esters on ^{3}H-PDBu binding

The structure–activity relationships for competition with ^{3}H-PDBu binding were examined to determine the specificity of the binding sites on the cells. MCF-7 cells were incubated at 37° for 30 min with 30 nM ^{3}H-PDBu in the absence or in the presence of various concentrations (10^{-9} M to 10^{-5} M) of phorbol derivatives. Figure 2 shows that the order of potency of the different phorbol esters in the inhibition of PDBu binding was: TPA, PDD > PDBu, PDBz > PDA, 4-*O*-met-TPA, αPDD > αPHR. This latter compound did not really compete until 10^{-5} M, the highest concentration tested. The IC_{50} values for binding inhibition were: TPA 35 × 10^{-9} M, PDD 12.5 × 10^{-9} M, PDBu 150 × 10^{-9} M and PDBz 220 × 10^{-9} M.

Effects of the different phorbol esters on cell proliferation

Using the same series of phorbol esters, we investigated the effect of various concentrations of the

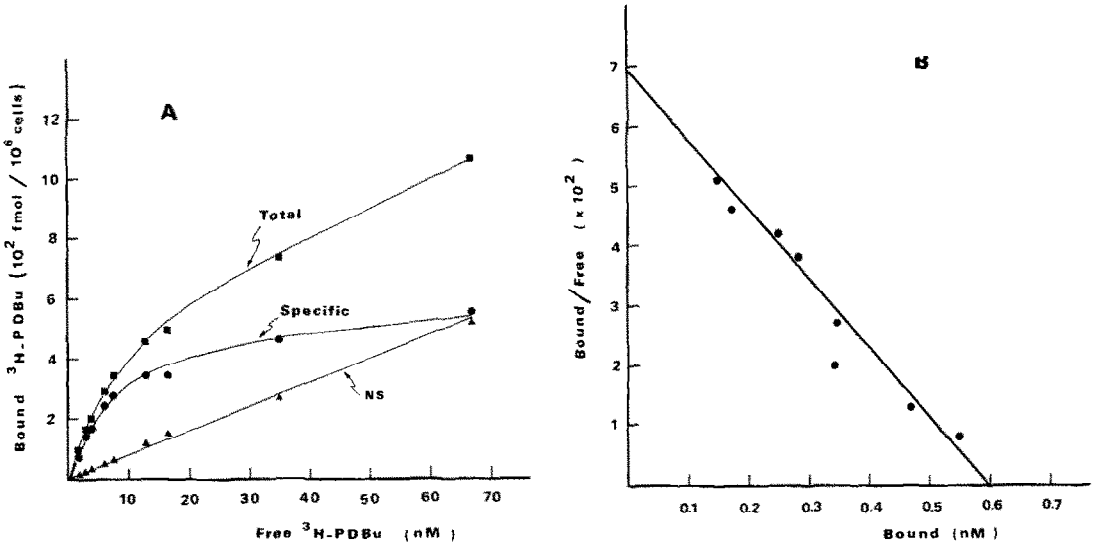

Fig. 1. Effect of ^{3}H-PDBu concentration on binding to MCF-7 cells. (A) Cells were incubated at 37° for 30 min with various concentrations of ^{3}H-PDBu in 1 ml of RPMI containing 0.1% BSA in the absence or in the presence of 10 μM unlabelled PDBu. Specific binding (●) was obtained by subtracting nonspecific binding (▲) from total binding (■). Data are the mean of duplicate measurements, from one experiment representative of three similar experiments. (B) Scatchard analysis of the specific binding data in A.

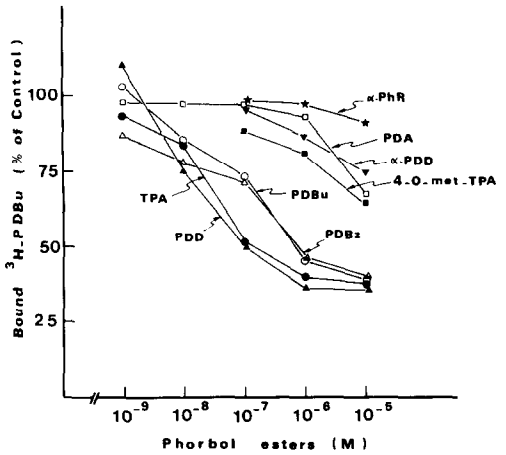

Fig. 2. Effects of various phorbol esters on ^{3}H-PDBu binding. Cells were incubated in 1 ml of RPMI containing 0.1% BSA with 30 nM ^{3}H-PDBu at 37° for 30 min with the indicated concentrations of the following phorbol derivatives: TPA (●), PDD (▲), PDBu (○), PDBz (△), PDA (□), 4-O-met-TPA (■), αPDD (▼), αPHR (★). All incubations were carried out with 0.5% DMSO as a final concentration. Data are expressed as a percent of the maximum binding obtained in the absence of unlabelled analogues. Each point represents the mean of 2 or 3 experiments (for each experiment, binding measurements were made in duplicate).

different derivatives (from 2×10^{-11} M to 2×10^{-7} M) on the MCF-7 cell proliferation during a 4-day culture period.

As shown in Fig. 3, the tumor promoter phorbol esters inhibit cell proliferation in a dose-dependent manner. The order of potency of the different derivatives was TPA > PDD > PDBu, PDBz > PDA. 4-*O*-met-TPA, αPDD and αPHR were ineffective at the concentrations tested. Higher concentrations for the various compounds were not used in this proliferation study because of their cell toxicity. The respective IC_{50} values for the inhibition of cell proliferation were: TPA 0.06×10^{-9} M, PDD 0.75×10^{-9} M, PDBu 2.4×10^{-9} M, PDBz 3.6×10^{-9} M, PDA 15×10^{-9} M. Although the IC_{50} for the inhibition of PDBu binding appears higher than the IC_{50} for the inhibition of cell proliferation, the relative potencies of the various phorbol esters in the inhibition of both parameters were quite similar.

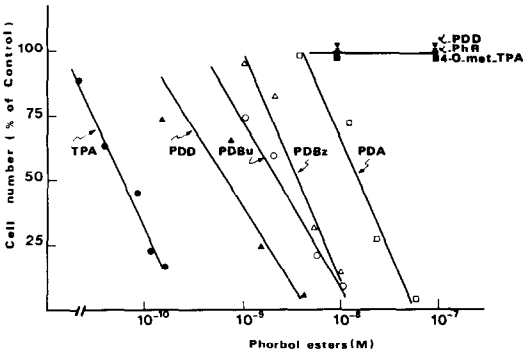

Fig. 3. Dose–response curves for the inhibiting effects of various phorbol ester derivatives on growth of MCF-7 cell after 4 days of treatment. Data are expressed as a per cent of the control cell number increase, between day 0 and day 4. Each point represent the mean of 2 to 3 different experiments. In each experiment, cell counting was performed on 3 separate dishes (the maximum variation of cell number did not exceed 10%). The number of control cells increased from $1–1.5 \times 10^5$ cells at day 0 to $0.8–1 \times 10^6$ cells at day 4. Symbols refer to those in Fig. 2.

DISCUSSION

We report here the presence of saturable and high specific phorbol ester binding sites in intact cultured MCF-7 cells. We also demonstrate a good correlation between the respective orders of potency of a series of phorbol ester derivatives to inhibit both PDBu binding and cell proliferation in the MCF-7 cell model. These data strongly support the implication of the phorbol binding sites in the mediation of the inhibitory action of tumor promoter phorbol esters in the MCF-7 cells. It is interesting to note that the order of potency that we have established parallels that observed *in vivo* in terms of skin tumor promotion. There is a lack of effect on both PDBu binding and cell proliferation for the phorbol derivatives that are not tumor promoters, i.e. 4α-phorbol.

However, active phorbol esters appear more effective in inhibiting cell proliferation than PDBu binding. This phenomenon has been described already in human myeloblastic leukemia ML-1 cells, where TPA has been shown to induce the inhibition of both ^{3}H- PDBu binding and cell proliferation, but with respective IC_{50} of 10^{-8} M and 10^{-10} M [22]. This may be due: (i) to the existence of phorbol ester spare receptors—in such a case, only a small fraction of phorbol ester binding sites, when occupied, could induce the maximal biological response; (ii) to the fact that the two parameters, PDBu binding and cell number, are not measured at the same time—as the phorbol esters are highly lipophilic, it may be possible that these compounds became concentrated in some membranous compartment during the course of the culture period.

As the PDBu binding affinity (K_d 9×10^{-9} M) is indeed comparable with the IC_{50} of PDBu for inhibiting cell proliferation (2.4×10^{-9} M), the second explanation seems more likely to account for the discrepancy observed. Such an hypothesis is further supported by the considerable sensitivity of the cells to TPA, when the inhibition of cell growth is considered. Indeed, this fact may be related to the extreme lipophilicity of TPA. More surprising is the relative efficiency of PDA in the inhibition of cell proliferation when compared to the very slight effect of this phorbol ester on ^{3}H-PDBu binding.

Protein kinase C has been suggested to be the receptor protein of phorbol esters. In rat brain, the relative effects of various phorbol derivatives on activation of purified protein kinase C and on ^{3}H-PDBu binding to the enzyme are closely correlated [18, 23]. The order of potency reported may be paralleled with the one we found in MCF-7 intact cells for the inhibition of PDBu binding and cell growth. Moreover, a recent report indicates a very close correlation between the dose–response curves

obtained for the biological effects of phorbol ester derivatives on rat adipocytes, and for those of the induced-protein kinase C translocation [24]. We have recently observed also in MCF-7 cells that active phorbol esters like TPA or PDBu induce both protein kinase C translocation, and specific protein phosphorylation while inactive phorbol derivatives like 4-*O*-met-TPA or 4α-phorbol do not (J. M. Darbon *et al.*, in preparation). The correlation we observed between the relative potencies of the various phorbol derivatives for inhibiting both PDBu binding and cell proliferation in the MCF-7 cells, suggests that tumor promoter phorbol esters may induce growth arrest in this cell system by the mediation of the protein kinase C.

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