0014-4754/93/010080-04$1.50 + 0.20

Two phorbol ester receptor affinities in partially transformed human urothelial cells and decrease of receptor binding in desensitized cells

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Received 25 May 1992; accepted 23 September 1992

Abstract. The presence of specific binding sites for phorbol esters was studied in a transformed but non-tumorigenic human urothelial cell line HCV-29 by assay of specific binding of ^{3}H-phorbol-12,13-dibutyrate (^{3}H-PDBu) to intact living cells. ^{3}H-PDBu bound specifically to HCV-29 cells in a saturable and competitive manner. Scatchard plot analysis of specific binding yielded a curved plot consistent with two binding sites with K_d of 11 nM and 102 nM, respectively. At saturation the corresponding PDBu binding capacities (B_{max}) were 8.8 pmol/10^6 cells (5.2×10^6 molecules bound per cell) and 2.8 pmol/10^6 cells (1.7×10^6 molecules bound per cell). ^{3}H-PDBu binding was displaced by biologically active phorbol ester tumor promoters such as 12-O-tetradecanoylphorbol-13-acetate (TPA) and mezerein, but not by tumor promoters such as L-tryptophan, anthranilic acid and sodium saccharin. In cells desensitized by pretreatment with 1 μg/ml (2 μM) TPA or PDBu for 24 h the level of binding was reduced to 28% of the level in non-exposed cells. The ability of desensitized cells to bind ^{3}H-PDBu was gradually restored within 5–6 days. At the same time the cells became sensitive to the morphological alteration induced by PDBu. This suggests that desensitization of HCV-29 cells is due to a decreased receptor-ligand binding capacity probably associated with down regulation of the phorbol ester receptors.
Key words. Phorbol ester receptor; desensitization; transformed human urothelial cell line; human urinary bladder carcinogenesis.

One of the most potent tumor promoter substances in experimental animal models is the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA). Normal human urothelial cells from primary cultures, and rodent urinary bladder mucosa contain specific binding sites for phorbol esters[1,2]. TPA has further been shown to activate protein kinase C in human urothelial cells[1]. TPA instilled into the rat urinary bladder led to an increase in bladder ornithine decarboxylase and induced transitional cell hyperplasia[2]. Phorbol ester tumor promoters thus seem useful compounds to study tumor promotion in urinary bladder carcinogenesis.

In our studies of chemical carcinogenesis using human urothelial cells[3–9], the effects of TPA were investigated using a transformed but non-tumorigenic human bladder epithelial cell line HCV-29[3]. HCV-29 cells responded to a single treatment with TPA by a rapid and transient change in morphology, by a rapid and transient increase in c-*fos* expression, and by the development of resistance to a renewed treatment with TPA that persisted for at least 24 h even in the absence of TPA. It has been speculated that the resistance was related to the number of receptors on the cells. In this communication, we report the results of characterization of phorbol ester binding in TPA responsive and resistant HCV-29 cells.

Materials and methods

Chemicals. Phorbol-12,13-dibutyrate (PDBu), 12-O-tetradecanoylphorbol-13-acetate (TPA), mezerein, L-tryptophan and anthranilic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium saccharin was purchased from Mecobenzon, Copenhagen, Denmark.

Phorbol-diesters and mezerein were dissolved in dimethyl sulfoxide (DMSO), L-tryptophan and anthranilic acid were dissolved in ethanol, and sodium saccharin was dissolved in distilled water. The stock solutions were stored at −20 °C and diluted in growth medium prior to use. The final concentration of solvents was 1‰ (v/v).

20-^{3}H-PDBu (spec. act. 12.5–15.8 Ci/mmol) was purchased from New England Nuclear (Boston, MA, USA).

Cell culture. The human urothelial cell line HCV-29 was derived from a non-malignant human bladder mucosa from a patient who was previously treated for bladder papillomata by irradiation[16]. HCV-29 has an infinite life span in vitro but the cells are non-tumorigenic in nude mice and non-invasive in vitro when co-cultured with normal tissue[10].

The cells were maintained in Fib 41b medium supplemented with 5% fetal bovine serum, 0.1 mM 7 non-essential amino acids, penicillin and streptomycin as previously described[10]. The medium was replaced every 3 days and the cells were subcultured once weekly.

Binding of ^{3}H-PDBu to cells. The binding of ^{3}H-PDBu to intact cells was assayed directly in Nunclon 4-well multidishes (NUNC, Roskilde, Denmark). Suspended cells (10^5 cells/ml medium) were inoculated into each

well 4–6 days before the experiment. For assay, the monolayer cultures were incubated in 1 ml growth medium containing ^{3}H-PDBu without unlabelled PDBu (total binding) or with unlabelled PDBu at a concentration 1000-fold in excess of ^{3}H-PDBu (non-specific binding). Following incubation at 37 °C and 5% CO_2 in air, aliquots of medium were removed from each well, and radioactivity was quantitated to determine the amount of free ^{3}H-PDBu. The cultures were placed on ice, the remaining medium was removed, and the cell layer was rinsed quickly 3 times with icecold growth medium. Cells were solubilized in 500 µl 0.1 N NaOH, and cell associated radioactivity was determined by liquid scintillation counting in a Beckman LS 1800 Scintillation Spectrometer in Ready-Solv aqueous counting cocktail. Specific binding was calculated as the total binding minus non-specific binding. Cell numbers were counted throughout all experiments and the amount of specifically bound ^{3}H-PDBu per 10^6 cells was calculated.

^{3}H-PDBu binding after exposure of cells to PDBu. Cells were desensitized by exposure to 1 µg/ml (2 µM) PDBu for 24 h. The medium was removed and the cells were washed twice for 1 h at 37 °C in growth medium. Control experiments using ^{3}H-PDBu showed that this washing procedure completely removed the radioactivity. After washing, ^{3}H-PDBu binding was determined as described above.

Results

Characteristics of binding. Binding of ^{3}H-PDBu to intact HCV-29 cells occurred rapidly (table). Binding was 40–50% of maximum after 2 min of incubation at room temperature. Incubation at 37 °C for 5 min sufficed to give maximal binding. No further increase in specific binding was observed at incubation times between 10 and 120 min.

Specific binding of ^{3}H-PDBu to intact HCV-29 was saturable (fig. 1). Scatchard plot analysis of specific binding yielded a curved plot consistent with heterogeneous binding. The plot was best fitted by two-sited linear regression analysis (fig. 2). The dissociation constants (K_d) derived were 11 nM and 102 nM, respectively. At saturation the corresponding PDBu binding capacities (B_{max}) were 8.8 pmol/10^6 cells (5.2×10^6 molecules bound per cell) and 2.8 pmol/10^6 cells (1.7×10^6 molecules bound per cell).

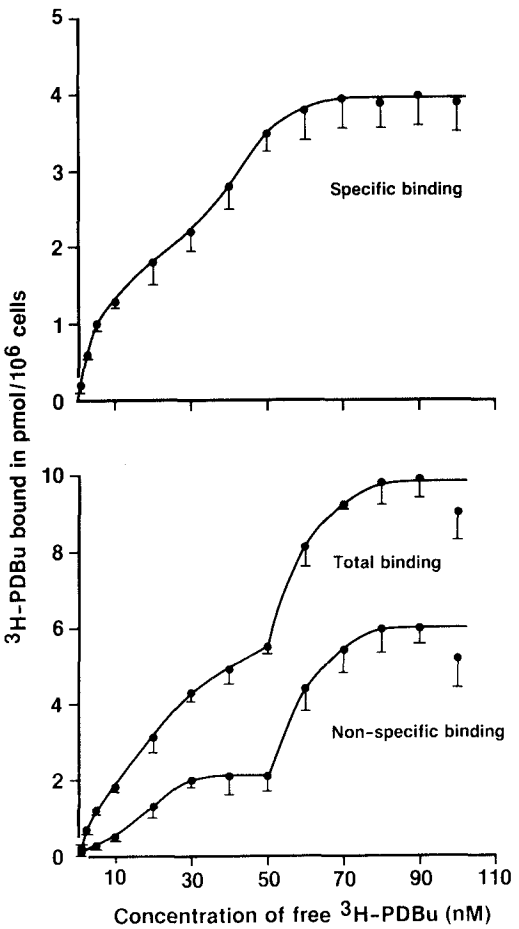

Figure 1. Binding characteristics of ^{3}H-PDBu to intact HCV-29 cells. Cells were incubated as described in 'Materials and methods' in the presence or absence of a 1000-fold excess of unlabelled PDBu. Specific binding was calculated by subtracting the non-specific binding from the total binding. Points are the mean of 5–10 experiments of duplicate cultures. Bars; SE.

Time course of ^{3}H-PDBu binding to intact monolayer of HCV-29 cells

Incubation time (min)	^{3}H-PDBu binding[a] (pmol bound/10^6 cells)
2	2.5
5	4.6
10	4.7
20	4.8
60	4.7
120	4.5

[a]Binding was determined in the presence of 60 nM ^{3}H-PDBu with 60 µM unlabelled PDBu. Each value is the mean of determinations carried out from quadruplicate assays. Variation less than 10%.

Binding of ^{3}H-PDBu was blocked by TPA, and by the related diterpene derivative mezerein (fig. 3). The concentrations required for 50% inhibition were lowest for TPA (10 nM) and highest for mezerein (104 nM) and spanned a range of 3 orders of magnitude. Binding of ^{3}H-PDBu was not inhibited by the nonphorbol ester tumor promoters, L-tryptophan, anthranilic acid and sodium saccharin.

Effect of TPA dose and exposure time on cell morphology. The morphological alterations induced by TPA in HCV-29 cells were independent of TPA concentration within the range of 10–1000 ng/ml (20 nM–2 µM), and independent of the exposure time within the range of 5 min to 24 h. Thus, exposure to 10 ng/ml (20 nM) for 5 min sufficed to induce a rapid and transient morphological response with a time-course similar to that seen

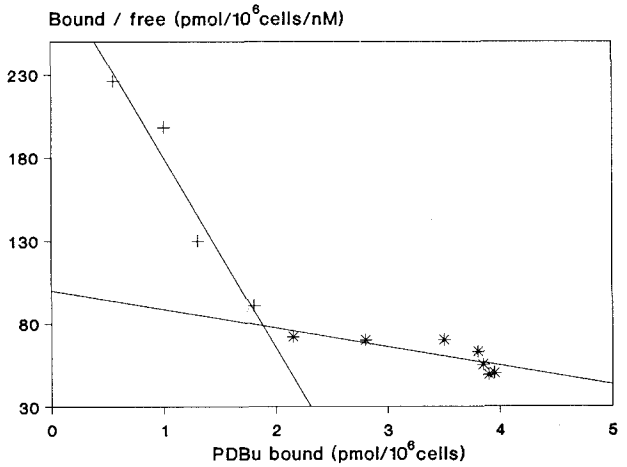

Figure 2. Scatchard plot analysis of specific binding. The lines were determined by linear regression.

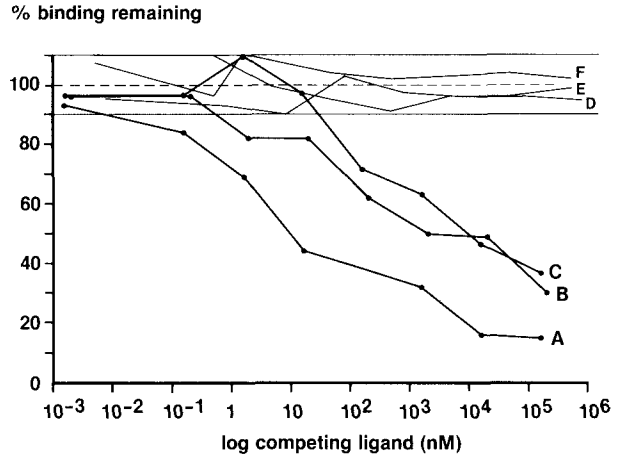

Figure 3. Competition of ^{3}H-PDBu binding by non-radioactive phorbol esters and non-phorbol tumor promoters. Competition experiments were carried out as described in 'Materials and methods' using a concentration of 20 nM ^{3}H-PDBu. A, TPA; B, PDBu; C, mezerein; D, sodium saccharin; E, L-tryptophan; F, anthranilic acid.

in cultures exposed to 1 μg/ml (2 μM) for 24 h [3]. At lower concentrations 1 ng/ml (2 nM) of TPA and shorter exposure time (less than 5 min) fewer cells responded morphologically, but the time course of the morphological alteration of the responsive subpopulation was similar to that seen in cultures exposed to higher concentrations or for longer time periods.

Neither L-tryptophan nor anthranilic acid or sodium saccharin affected the morphology of the cells when tested at concentrations of 10 ng/ml–1 mg/ml.

Binding of ^{3}H-PDBu in desensitized cells. The inability of HCV-29 cells to respond morphologically to a renewed treatment with TPA or PDBu lasted for 4–5 days when challenged with 1 μg/ml (2 μM) PDBu, but 6–7 days when challenged with 20 ng/ml (40 nM). In order to investigate whether the transient desensitization of the cells to PDBu induced morphological alter-

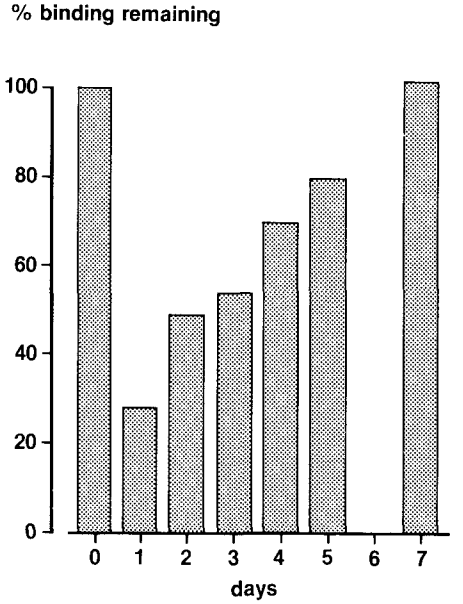

Figure 4. Specific binding of ^{3}H-PDBu to desensitized human urothelial cells. Cells were desensitized by pretreatment with 1 μg/ml PDBu for 24 h. The medium was removed and the cells washed thoroughly (see 'Materials and methods'). Following various periods of recovery, specific binding was determined in the presence of 60 nM ^{3}H-PDBu with or without 60 μM PDBu.

ations might be correlated to a decrease in binding of ^{3}H-PDBu, the level of binding of ^{3}H-PDBu in cells desensitized by pretreatment with 1 μg/ml (2 μM) of PDBu for 24 h was investigated. After 24 h the level of binding was reduced to 28% of the level in cells not previously exposed to PDBu (fig. 4). After further recovery, the level of binding gradually increased. At day 5, when the cells were desensitized to the induction of morphological alteration induced by 20 ng/ml (40 nM) PDBu but responsive when treated with 1 μg/ml (2 μM), the binding level was 80%. At day 7 when the cells had regained the responsiveness to PDBu concentrations of both 20 ng/ml (40 nM) and 1 μg/ml (2 μM), the level of binding was 102%.

Discussion

By assaying the binding of ^{3}H-PDBu to intact cells we have demonstrated that partially transformed human urothelial cells contain specific and saturable binding sites for phorbol esters. The specific binding of PDBu could be displaced by other biologically active phorbol ester tumor promoters. The concentration required for 50% inhibition of ^{3}H-PDBu binding was lowest for TPA and highest for mezerein indicating a correlation between tumor promoting activity of these compounds and affinity for phorbol ester binding sites[1]. ^{3}H-PDBu binding to the cells was not displaced by the rat bladder tumor promoters sodium saccharin, L-tryptophan and antranilic acid. These results are in agreement with previous reports[1,2].

Analysis of the binding of PDBu to HCV-29 cells suggested the existence of two phorbol ester receptors. Two classes of binding sites for PDBu have previously been described in cultured primary normal human keratinocytes and melanoma cultures[11] but not in human urothelial cells[1]. Using ^{3}H-TPA Verma et al.[1] demonstrated a single class of high affinity binding sites (K_d 0.56 nM) in primary cultures of normal human transitional cells. At saturation, the binding capacity of these cells was 2.37 pmol/10^6 cells corresponding to 1.43×10^6 binding sites per cell. Thus the immortalized, non-tumorigenic human urothelial cell line HCV-29 differs from normal transitional cells by a higher level of phorbol ester binding sites and by the presence of two phorbol ester receptors with lower affinities. It is not known if these differences between HCV-29 cells and normal transitional are associated with the transformation of normal cells into immortalized, non-tumorigenic cells, or whether it reflects a difference between long-term and short-term cultured cells or experimental design.

We have previously shown that HCV-29 cells responded to a single treatment of 1 μg/ml (2 μM) TPA by a rapid and transient change in morphology and in c-*fos* expression[3]. The induction of c-*fos* was dependent on the concentration of TPA, requiring at least 80 ng/ml (160 nM)[4]. In the present study we have shown that the morphological response was independent of the concentration of TPA within the range of 10–1000 ng/ml (20 nM–2 μM). This suggests the existence of functional phorbol ester receptor heterogeneity in HCV-29 cells and it might be speculated that the two different receptor affinities are correlated to changes in morphology and changes in c-*fos* expression.

HCV-29 cells which had been exposed to PDBu for 24 h became refractory to the PDBu induced morphological alteration. At the same time they showed a decreased capacity to bind PDBu. After removal of PDBu and a thorough washing of the cells in order to remove residual PDBu, the ability to bind the ligand was gradually restored within 6–7 days. At the same time the cells became sensitive to the morphological alterations induced by PDBu. Desensitization, decrease in binding and the reversal of both phenomena thus occur with similar kinetics. This suggests that desensitization of HCV-29 cells was due to a decreased receptor-ligand binding capacity probably associated with down regulation of the phorbol ester receptor[12–20].

Human urothelial cell lines propagated in vitro in our laboratory can be classified into 3 grades of transformation (TGrI, TGrII and TGrIII) according to their in vitro characteristics[10]. TGrI cell lines have a finite life span, TGrII cell lines are immortal but non-tumorigenic while TGrIII cell lines are immortal and tumorigenic. Slightly transformed TGrI cell lines, premalignant TGrII cell lines and malignant TGrIII cell lines all differ from early cultures of normal urothelium (TGr0) by a prolonged lifespan[10]. It has been hypothesized that these 3 categories of transformed cell lines represent different and possibly successive stages of transformation in a multistage carcinogenic process[10]. Transformation of premalignant TGrII cell lines into malignant TGrIII cells support this hypothesis[21,22]. Since the cell line HCV-29 classified as TGrII contain specific receptors for phorbol ester tumor promoters, these cells may also be a useful tool to study tumor promotion in human urinary bladder carcinogenesis following intermittent treatments with various concentrations of TPA.

Acknowledgment. The technical assistance of Jacqueline Schmidt is gratefully acknowledged. The work was supported by grants from the Danish Cancer Society.

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