Effects of Phorbol 12-Myristate 13-Acetate and Cortisol Interaction on Steroid-Binding Capacity in the Rat

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The specificity of the cortisol-receptor protein is examined in plasma and liver cytosol of rats. Phorbol 12-myristate 13-acetate does not inhibit the binding of cortisol to transcortin, nor does it affect the binding capacity of dexamethasone to the intracellular glucocorticoid receptor, but, by interacting with the cortisol molecule, it interferes with hormone-mediated processes in the cell.

Croton oil and particularly its active principle the phorbol diesters are known to be some of the most potent tumour promoters when used in association with carcinogenic initiators (Hecker & Schmidt, 1974). The application of phorbol diesters is known to affect the activities of certain enzyme systems (Colburn *et al.*, 1975; O'Brien *et al.*, 1975; Murray & Frosco, 1977), cellular oxidative metabolism (De Chatelet *et al.*, 1976), cyclic AMP and cyclic GMP concentrations (Belman & Troll, 1974; Goldstein *et al.*, 1975; Trosko *et al.*, 1975; Verma *et al.*, 1976), RNA, DNA and protein concentrations (Rohrschneider *et al.*, 1972; Raick, 1973; Balmain, 1976), ATP synthesis, phosphorylation of histones (Raineri *et al.*, 1973) and certain membrane functions (Van Duuren *et al.*, 1976). However, most of these effects seem to be very similar to the cellular modifications provoked by the main initiators, such as the carcinogenic polycyclic hydrocarbons (Atri *et al.*, 1972; De Loecker *et al.*, 1972; Langenbach & Kuszynski, 1975). Although the mechanism of carcinogenesis is far from understood, initiators are considered to be responsible for specific molecular interactions leading to somatic mutations, whereas promoters possibly act at a subsequent metabolic stage, as yet unexplained (Ryser, 1971). As glucocorticoids suppress the promoter effect of phorbol esters and even protect against carcinogenesis (Viaje *et al.*, 1977), direct molecular interaction between cortisol and phorbol 12-myristate 13-acetate is suggested to take place, on the basis of structural analogy (Wilson & Huffman, 1976). In an attempt to explore these possible molecular interactions, the effects of phorbol diesters on cortisol, on cortisol-plasma binding and on glucocorticoid-receptor molecules have been examined.

Materials and Methods

Plasma and liver of locally inbred female Wistar R rats weighing 200 g were used throughout these
Vol. 184

experiments. The animals were decapitated and the tissues were excised, dissected, rinsed in cold 0.9% NaCl and used immediately or packed in plastic containers (Greiner granules) and frozen by immersion in liquid N₂. Plasma was used either undiluted or diluted 100-fold with TED buffer, pH 7.4 [10 mM-Tris/HCl (Merck)/1.5 mM-EDTA (Calbiochem)/0.5 mM-1,4-dithio-PL-threitol (Fluka)]. The weighed tissue was broken up at -196°C in a steel mortar and subsequently homogenized in a Potter/Elvehjem homogenizer in 3 vol. of TED buffer, pH 7.4, for assay of cortisol or dexamethasone. The homogenate was centrifuged at 105000g (International Ultracentrifuge B60, swinging-bucket SB 405) for 70 min and the supernatant cytosol removed. The receptor assays were carried out in duplicate, as described by Koenders *et al.* (1977). Cooke microtitre plates (Greiner) (V-shaped) each with 8×12 cuvettes of volume 0.2 ml were used. All further preparations were done in ice and started by transferring (micropipettes; Socorex-Hamilton-Brand) 5 µl of TED buffer into eight cuvettes. To determine aspecific steroid binding, unlabelled cortisol (Merck, Sharp and Dohme), at a concentration that exceeded the highest radioactive tracer concentration by 1000-fold, and phorbol 12-myristate 13-acetate (Sigma) were added to two cuvettes to block the receptor present in the cytosol. Cytosol (50 µl) was subsequently added to two cuvettes with and to two cuvettes without unlabelled steroid and preincubated at 4°C for 30–60 min to allow interaction between the receptors in the cytosol and the unlabelled steroids. Another four cuvettes contained only 5 µl of buffer. Finally 5 µl (containing 0.1–20 nmol) of labelled steroid [either [1,2,6,7-³H]cortisol (sp. radioactivity 80–105 Ci/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) or [1,2(n)-³H]dexamethasone (sp. radioactivity 25 Ci/mmol; The Radiochemical Centre)] was added to the samples. The titre plates were covered with Parafilm to reduce evaporation and incubated at 4°C for 16–20 h on a vibrating

micro-shaker (Dynatech). After incubation, two cuvettes containing no cytosol received 100 μ l of DCC suspension [TED buffer/0.25% charcoal/0.025% dextran (grade C; BDH Biochemicals)] to precipitate all the unbound radioactive tracer. The radioactivity recovered in the supernatant never exceeded 2% of the initial dose. This fraction, however, was important for correction of the total binding, and is designated the DCC blank. As there were two other cytosol-free cuvettes that did not receive DCC suspension, the radioactivity remaining in the solution represents the total added tracer amount. The next two cuvettes, containing cytosol and radioactive steroid and representing the total bound tracer (specific and aspecific binding), and the two cuvettes that also contained the 1000-fold concentration of unlabelled steroid and represent aspecific binding all received 100 μ l of DCC suspension. Addition of DCC suspension to remove the unbound steroids took place with constant shaking followed by centrifugation for 10 min at 1000g (International, size 2, model K, rotor 240 with microtitre plate carrier). To obtain a constant final volume of 160 μ l, the cuvette contents were made up with the corresponding buffer where necessary.

After centrifugation, 100 μ l of supernatant was transferred to mini-vials 3 (Lumac Systems A.G.) containing 1 ml of scintillation liquid 'Aqua Luma' (Lumac Systems A.G.) and counted for radioactivity for 10 min in a liquid-scintillation spectrometer

(Nuclear-Chicago, 720 series). After correction for background, DCC blank and quenching, the radioactive counts were converted to receptor concentrations and binding constants, and subsequently derived from the Scatchard plots (Scatchard, 1949) and expressed as concentrations of receptor (fmol per vial, per mg of protein or per mg of tissue) and as binding constants, K_D , of the receptor. Tissue protein concentrations were determined by the Folin-phenol method (Lowry *et al.*, 1951) and were about 20 mg of protein/ml of liver cytosol.

Results

Glucocorticoid receptors were determined quantitatively in rat liver. Interference with transcortin was taken into account and excluded by simultaneous receptor determinations in plasma. The synthetic glucocorticoid dexamethasone was used instead of cortisol to avoid contaminating binding with transcortin. No detectable binding of dexamethasone in plasma occurred, whereas liver incubations showed specific binding capacities with values of 20.5 fmol/mg of protein and a dissociation constant, K_D , of 1.0 nmol (Figs. 1a and 1b). The specific binding of cortisol to transcortin was measured in undiluted rat plasma (Fig. 2a). The initial part of the saturation curve represents the specific binding of labelled cortisol to rat plasma, which differs from the intracellular glucocorticoid binding, as confirmed by the

Fig. 1. Specific binding of dexamethasone in cytosol

(a) Liver (●) and plasma (○) from Wistar R rats were homogenized with 3 vol. of TED buffer at pH 7.4 (20 mg of protein/ml). The 105 000g supernatant was incubated in Cookes microtitre plates with 0.1–1.0 nM-[1,2(n)-³H]dexamethasone. Aspecific binding, measured by the initial addition of 1 nmol of unlabelled cortisol, was deducted. (b) The final concentration of dexamethasone bound was determined by Scatchard analysis. Only the liver cytosol exhibited a saturable high specific binding.

Fig. 2. *Specific binding of cortisol to transcortin*

(a) Undiluted plasma of Wistar R rats was incubated with [1,2,6,7-³H]cortisol for 16 h in Cookes microtitre plates. The concentrations of the labelled steroid varied from 0.5 to 20.0 nM. The effects of aspecific binding were corrected for by the simultaneous addition to the incubation medium of 1 μ M-unlabelled cortisol. (b) In the same circumstances, labelled dexamethasone was incubated. The concentrations of [1,2 (n)-³H]dexamethasone varied from 0.5 to 1.0 nM.

fact that dexamethasone binding is absent from plasma (Fig. 2b).

In order to evaluate the effect of the phorbol ester on the binding of cortisol to transcortin, different concentrations were incubated with plasma and labelled cortisol. Simultaneously the aspecific binding was measured by adding a 500-fold excess of cortisol to the incubation medium. The total binding capacity of labelled cortisol in plasma diluted 100 times was not significantly affected by the addition of the phorbol ester in concentrations varying from 1 μ M to 1 nM (Fig. 3). To establish possible binding of the phorbol ester to cortisol, they were preincubated together for 1 h at 4°C in the relative concentrations phorbol ester/cortisol varying from 0 to 1000. This mixture was subsequently incubated with liver cytosol and labelled dexamethasone. If cortisol is not affected by the phorbol ester, the different incubations should produce identical binding curves, whereas, if it is affected, the binding curves obtained with different phorbol ester concentrations should vary between those representing aspecific binding and those representing total binding. Indeed the inhibitory effect of a 1000-fold excess of unlabelled cortisol was decreased by the phorbol ester. This effect initially occurred at the lowest labelled-dexamethasone concentrations and was almost proportional to the phorbol ester concentrations (Figs. 4a and 4b). The phorbol ester, however, does not interfere with the binding of dexamethasone to the glucocorticoid receptor, as the binding was not affected, even after the addition of increasing amounts of the phorbol ester to the incubation medium (Fig. 5).

Fig. 3. *Effects of different phorbol ester concentrations on the binding of cortisol to transcortin*

Plasma diluted 100-fold was incubated with the phorbol ester (1 μ mol, 10 μ mol, 0.1 mmol, 1 mmol) or with unlabelled cortisol (1 μ mol) for 1 h. Subsequently, labelled cortisol was added and incubation was continued overnight at 4°C. ●, Total bound labelled cortisol, without the addition of the phorbol ester or unlabelled cortisol (mean $\pm$ s.d. for six experiments); Δ , bound cortisol after addition of 1 mmol of the phorbol ester; $\blacktriangle$, bound cortisol after addition of 0.1 mmol of the phorbol ester; $\blacksquare$, bound cortisol after addition of 10 μ mol of the phorbol ester; $\square$, bound cortisol after addition of 1 μ mol of the phorbol ester; $\circ$, bound cortisol with addition of 1 μ mol of unlabelled cortisol.

Discussion

Although adrenalectomy increases the specific cytoplasmic binding sites for corticosteroids in liver,

Fig. 4. Effect of the phorbol ester on cortisol binding

(a) To liver cytosol, obtained and treated as described (Materials and Methods section) containing 20mg of protein/ml, was added a mixture of cortisol and the phorbol ester at phorbol ester/cortisol ratios of 0, 0.1, 1, 10, 100 and 1000. Subsequently, [1,2(n)-³H]dexamethasone (0.14–0.78 nmol/litre of incubation medium) was added. ●, Total binding of labelled dexamethasone to its receptor (mean $\pm$ S.D. for six experiments); ○, binding of dexamethasone when 1000-fold excess of cortisol was added; □, binding of dexamethasone with 1000-fold excess of cortisol (1 μ M) and 0.1 μ M-phorbol ester; ▲, binding of dexamethasone with 1000-fold excess of cortisol (1 μ M) and 1 μ M-phorbol ester; ■, binding of dexamethasone with 1000-fold excess of cortisol (1 μ M) and 10 μ M-phorbol ester; △, binding of dexamethasone with 1000-fold excess of cortisol (1 μ M) and 1 mM-phorbol ester. (b) Specific binding, determined as the difference between total binding of dexamethasone with or without a 100-fold excess of unlabelled cortisol (100%), was compared with the simultaneous addition of different concentrations of the phorbol ester. These results were obtained with the same data as for (a). ○, Total binding of dexamethasone in the absence of cortisol minus aspecific binding obtained in the presence of excess cortisol; □, ■, △, ▲, total binding minus aspecific binding in the presence of the phorbol ester and cortisol. The symbols are as for (a).

intact animals were used to avoid modifying physiological conditions. In these circumstances, however, the ratio between aspecific and specific binding sites of cortisol and dexamethasone is proportionally higher. As multiple binding sites may interact with cortisol, the linearity of the Scatchard plots indicates that in the present experiments only one specific binding site is to be considered (Agarwal *et al.*, 1977a,b).

If the phorbol ester does interfere with cortisol metabolism, three possible interaction sites should be taken into account, the plasma-binding (transcortin) concentration, the intracellular binding (glucocorticoid-receptor binding) site and the direct interaction between the phorbol ester and cortisol molecules. The latter possibility in particular seems interesting, as analogous stereochemical characteristics between the two molecules have been described (Wilson & Huffman, 1976) and as the phorbol ester

seems to be recognized as cortisol in the radioimmunoassay (Smythies *et al.*, 1975). As plasma contamination always interferes with intracellular cortisol binding, this is prevented by using the synthetic corticosteroid dexamethasone, which exhibits no binding capacity in plasma. Thus the dexamethasone assay constitutes an effective method for analysing the possible interactions of the phorbol ester and the intracellular receptor. Cortisol assay as such is reserved for plasma binding studies. Finally, a possible specific interaction between the phorbol ester and cortisol molecule is examined by analysing the comparative effects of cortisol, with or without the phorbol ester, on dexamethasone-receptor binding.

If the phorbol ester does bind to cortisol, the amount of cortisol bound to its receptor should be decreased, and proportionally more dexamethasone should be attached to the receptor. Although the

Fig. 5. Effect of the phorbol ester on the binding of [1,2(n)-³H]dexamethasone to its receptor

Liver cytosol (20 mg of protein/ml) was incubated as described in the text at 4°C with 1 μM-cortisol or with 0.1 μM- to 1 mM-phorbol 12-myristate 13-acetate. Subsequently, labelled dexamethasone was added in concentrations varying from 0.14 to 1.1 nmol. ●, Total binding of [1,2(n)-³H]dexamethasone in the absence of unlabelled steroid (mean ± S.D. for six experiments). Binding of [1,2(n)-³H]dexamethasone in the presence of 0.1 μM- (□), 1 μM- (■), 10 μM- (▲), 0.1 mM- (▽) or 1 mM- (Δ) phorbol 12-myristate 13-acetate. ○, Binding of dexamethasone in the presence of 1 μM-cortisol (mean ± S.D. for six experiments).

phorbol ester does not interfere with the binding of cortisol to transcortin, and does not prevent dexamethasone from binding to the intracellular glucocorticoid receptor, it affects the inhibitory effect of cortisol on the binding of dexamethasone to its receptor. Indeed, even when small quantities of the phorbol ester are incubated with cortisol, the inhibitory effect of cortisol is lower with lower dexamethasone concentrations, as a result of the higher affinity of dexamethasone for the receptor than for the cortisol. This explains the fact that even small doses of the phorbol ester affect cortisol-dependent processes. This phorbol ester-cortisol relationship appears to be particularly specific, as no such effect is observed when [17α-methyl-³H]-methyltrienolone (R1881) or [2,4,6,7-³H]oestradiol is incubated with the phorbol ester and respectively testosterone and oestradiol-17β. The interaction could be responsible for the tumour-promoting effect of the phorbol ester (Wilson & Huffman, 1976).

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