Ultraviolet-B (290–320 nm)-Irradiation Inhibits Epidermal Growth-Factor Binding to Mammalian Cells*

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Mitogens, such as polypeptide growth factors and phorbol ester tumor promoters, act by binding to specific receptors and inducing a pleiotrophic response in cultured mammalian cells, which results in the induction of cellular proliferation.

An early effect of such agents is the inhibition of binding of epidermal growth factor (EGF) to its receptor. Ultraviolet radiation has also been shown to induce a proliferative response in vivo and in vitro and to act as a tumor promoter in animal skin. We, therefore, examined the effect of ultraviolet radiation (UVB — 290–320 nm) on EGF binding to cells in culture. We found that UVB (100–300 J/m^2) induced a rapid, dose-dependent inhibition of EGF binding in a mouse fibroblast cell line, which resulted from a decrease in both number and affinity of binding sites. Phosphorylation of the EGF receptor by protein kinase C (PKC) is not likely to be the mechanism for inhibition, since UVB treatment did not result in PKC activation or modulation of phorbol diester binding. *J Invest Dermatol 92:617–622, 1989*

Ultraviolet radiation (UV) induces a complex response in human and animal skin including keratinocyte damage with an acute inflammatory response followed by a repair phase with cellular proliferation leading to tissue hyperplasia [1–3]. In addition, UV can act as a complete carcinogen in animal skin, suggesting that it may act as both an initiator and a promoter of tumorigenesis [4,5]. The mechanism of the acute response and its relationship to carcinogenicity of ultraviolet radiation are undefined.

A number of agents have been identified which induce proliferation in cell-culture systems including platelet-derived growth factor (PDGF) and fibroblast-derived growth factor (FDGF); mitogenic hormones, like vasopressin; and various tumor promoters including the phorbol esters and teleocidin [6–9]. The mechanism by which these agents induce DNA synthesis and mitogenesis and the relationship of those events to the loss of control of growth associated with carcinogenesis have been the subject of intense research activity. Such investigations have yielded evidence that these agents induce cellular changes through a system of second messengers. Many of these changes occur at the membrane level, resulting in the synergistic activation of cyclic-AMP and protein kinase-C pathways. An early event in this mitogenic signaling includes a rapid modulation of the binding of EGF to specific surface receptors on cells treated with such factors, and this alteration of EGF binding has been assayed as a marker for activation of such signaling systems [10].

Since UV radiation has been shown to stimulate cellular proliferation both in intact skin and cells in culture [3,11], we have been interested in investigating the effect of such radiation on early membrane events. The present studies present evidence that UVB (290–320 nm) irradiation of mammalian cells modulates EGF binding. This effect is probably due to a mechanism other than PKC-mediated phosphorylation of the EGF receptor.

MATERIALS AND METHODS

Reagents and Media [^{125}I]EGF (125–165 uCi/ug), [^{3}H]Phorbol dibutyrate (PDBu) (16 Ci/mmol), and [γ^{32}P] ATP (30 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, MA). Receptor grade EGF and PDBu, histone III-S, ATP, bovine serum albumin, phenylmethylsulfonyl fluoride (PMSF), Tris hydrochloride and DEAE-Sephacel were purchased from Sigma Chemical Co. (St. Louis, MO). Whatman phosphocellulose paper (Grade P81) was obtained from Fisher (Springfield, NJ), phosphatidylserine (PS) from Avanti Polar Lipids (Birmingham, AL), and 12-0-tetradecanoylphorbol-13-acetate (TPA) from LC Services. Leupeptin was a generous gift of the US-Japan Cooperative Cancer Program.

Dulbecco's Modified Eagle's Medium (DMEM), penicillin, streptomycin, Hank's buffered salt solution without phenol red, Ca^{+2}, Mg^{+2} (HBSS w/o Ca^{+2}), and Hank's buffered salt solution without phenol red (HBSS w/Ca^{+2}) were all obtained from Gibco Laboratories. Calf serum and plastic tissue culture dishes were from Flow Laboratories (Grand Island, NY).

Cell Culture Mouse fibroblast C3H/10T1/2 cells were used between passages 9 and 15 and were maintained in DMEM supple-

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Abbreviations:
DMEM: Dulbecco's modified Eagle's medium
EGF: epidermal growth factor
FDGF: fibroblast-derived growth factor
HBSS w/CA^{+2}: Hank's buffered salt solution without phenol red
HBSS w/o Ca^{+2}: Hank's buffered salt solution without phenol red, Ca^{+2}, and Mg^{+2}
KM: keratinocyte medium
[^{3}H] PDBu: [^{3}H] phorbol dibutyrate
PDGF: platelet-derived growth factor
PKC: protein kinase C
PMSF: phenylmethylsulfonyl fluoride
PS: phosphatidylserine
TPA: 12-0-tetradecanoylphorbol-13-acetate
UVB: ultraviolet-B radiation

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mented with 10% calf serum, penicillin (75 units/ml), and streptomycin (25 ug/ml).

Light Source and Irradiation Irradiation was performed on monolayer cultures with lids removed, utilizing a light source composed of 6 Phillips Fluorescent Sun Tubes (Irradiance = 12 W/m^2 measured with an International Light Research Radiometer IL700 with a SEE 1240 probe). Control cells in HBSS w/Ca^{+2} were placed in the field of irradiation with opaque covers (sham irradiated). The irradiation procedure was begun by removing growth media from dishes, washing twice with HBSS w/o Ca^{+2}, and covering the cells with a small amount of HBSS w/Ca^{+2}. Exposure time was 0.13 s per J/m^2, so that a dose of 600 J/m^2 would require an exposure time of 80 s. No detectable heat was produced during irradiation. After irradiation, medium without serum was added, and culture dishes were incubated at the temperatures and lengths of time indicated for each experiment.

Ligand Binding Assays The effect of UVB on EGF binding was assayed at subconfluence in 6-well or 35-mm tissue-culture dishes. Binding to intact cells was initiated after treatment by replacing the incubation medium with binding assay buffer (AB) (1 mg/ml bovine albumin in DMEM/PBS, 2:1) containing [^{125}I] EGF. Binding was terminated by placing culture dishes on ice and washing rapidly with three 1-ml aliquots of ice-cold AB. Cells were solubilized in phosphate-buffered saline containing 0.8% Triton X-100, 0.2% EDTA, and 0.25% trypsin. Plates were then washed twice with 1% sodium dodecyl sulfate. Solubilization and wash solutions were combined and counted in Hydrofluor scintillation fluid (National Diagnostics, Highland Park, NJ).

Data are expressed as specific binding, which was obtained by subtracting ligand bound nonspecifically (determined in parallel dishes which contained 100 fold excess of unlabelled EGF) from total ligand bound. Values calculated for receptor number and affinity from Scatchard plots were obtained by standard methods [12–15].

The cell surface-bound [^{125}I] EGF was measured by the method of Haigler et al. [19]. Cells were washed once with ice-cold PBS, incubated with 0.2 M acetic acid, 0.5 M NaCl for 5 min at 4°C, and washed once more with the same buffer. The washes were combined, and acid-soluble and cell-bound radioactivity was determined.

PDBu binding was assayed in a similar fashion, as described previously [16]. Cells were grown in 6-well tissue-culture dishes until subconfluent. Binding was carried out by first preincubating cells in assay buffer for 45 min, then treating the cells with UVB, sham-irradiation, TPA or DMSO, as described above. Assay buffer containing [^{3}H]PDBu (12 nM final concentration) was added to the wells to initiate binding. Wells were incubated for 45 min at 37°C, then binding was terminated by the procedure for EGF binding, as described above. Parallel wells were incubated with an additional 50 μM PDBu to measure nonspecific binding. All data points were calculated from triplicate wells for both total and nonspecific binding.

Protein Kinase C Assay Measurement of protein kinase C activity distribution between cytosolic and membrane compartments was modified from procedures described elsewhere [17]. 10-cm dishes of C3H/10T1/2 cells were irradiated, as described above, with selected doses of UVB. Irradiated and sham-irradiated cells were incubated at 37°C for the lengths of time post irradiation indicated in *Results*. Cells were treated with TPA (250 ng/ml) for 45 min at 37°C as a positive control.

Cells were harvested after incubation by washing with ice-cold PBS and scraping into PBS with a rubber policeman. After centrifugation at 1000 × *g*, cell pellets were homogenized in ice-cold Buffer A (20 mM Tris-HCl, 5 mM EGTA, 2 mM EDTA, 0.25 mM PMSF, 5 mM B-mercaptoethanol, and 20 ug/ml leupeptin) by 30 strokes in a Dounce homogenizer. All subsequent steps were performed at 4°C. The homogenate was centrifuged at 100,000 × *g* for one h. The supernatant was removed, placed on ice and represented "cytosolic" protein. The pellet was resuspended in Buffer A with 1% Triton and stirred vigorously for 45 min. The suspension was centrifuged at 100,000 × *g*, and the supernatant was removed. This contained "membrane" protein. Cytosolic and membrane fractions were loaded onto DEAE-Sephacel columns equilibrated with Buffer A, and partially purified PKC was eluted with 0.3 M NaCl in Buffer A. The eluate was concentrated by ultrafiltration using an Amicon YM-30 membrane. The resultant solutions were used to determine PKC activity according to established methods [18].

PKC activity was defined as the phosphotransferase activity which occurred in the presence of Ca^{+2}, phosphatidylserine (PS), and TPA after subtraction of activity which occurred in the absence of cofactors. The standard reaction mixture (0.12 ml) contained 20 mM Tris-HCl at pH 7.5, 10 mM $MgCl_2$, 1 mM $CaCl_2$ (or 1 mM EGTA), 50 uM TPA (or no TPA), 30 ug/ml PS (or no phospholipid), 70 uM [^{32}P] ATP (300–500 cpm/pmol), 0.67 mg/ml histone III-S, and 4–12 ug cell protein.

RESULTS

Effect of UVB-irradiation on EGF Binding When binding was assayed immediately after irradiation, UVB treatment of C3H/10T1/2 cells resulted in a dose-dependent inhibition of EGF binding within a range of 100–300 J/m^2 (Fig 1). The level of binding of the cells treated with 300 J/m^2 was approximately 25% of that of the control cells. Higher doses of UVB failed to increase this inhibition.

The inhibition was not due simply to UVB-induced cytotoxicity, since a trypan-blue assay revealed no difference in short-term viability between irradiated and control cells. Number of cells per dish was determined at 0,1,2,4,6,8,16 and 24 h postirradiation. Only at 24 h was a decrease (30%) apparent in the number of cells on irradiated dishes compared to sham-irradiated cells. In addition, pilot studies revealed that UVB had no effect on nonspecific binding.

To examine the time course of this inhibition, we performed binding assays at intervals after irradiation with 300 J/m^2 (Fig 2).

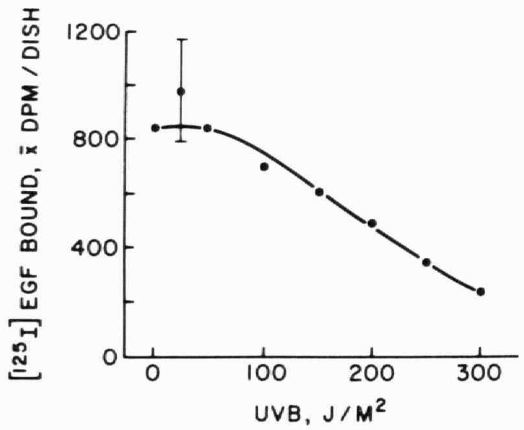

Figure 1. The effect of UVB radiation on specific [^{125}I]EGF binding to C3H/10T1/2 cells in culture. Subconfluent cultures were washed with Hank's buffered salt solution without Ca^{+2} (HBSS w/o Ca^{+2}), covered with HBSS w/Ca^{+2}, and immediately irradiated with selected doses of UVB at room temperature. Irradiation and sham irradiation were performed, as described in *Materials and Methods*. Immediately after irradiation, binding was initiated by the addition of assay buffer containing [^{125}I]EGF (final concentration of 50 pM) and continued for 50 min at 37°C, as described previously [16]. Assays for total binding were always performed in parallel with assays for nonspecific binding, as indicated in *Materials and Methods*. Nonspecific binding never exceeded 20% of total binding, and was not altered by irradiation. Data represent means of triplicate determinations; ±SEM. SEMs are less then 10%, unless shown.

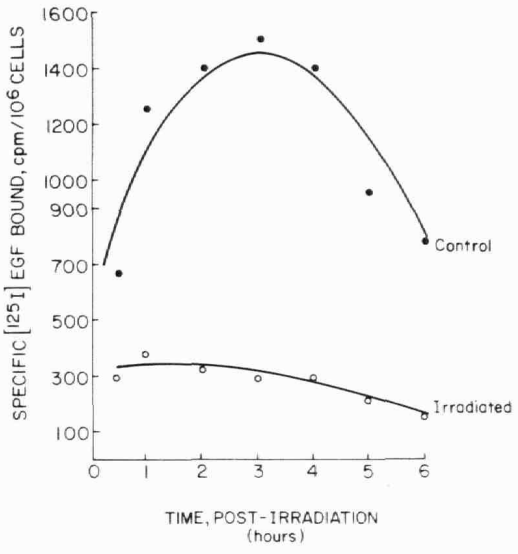

Figure 2. The time course of UVB radiation-induced reduction in [^{125}I]EGF binding to intact C3H/10T1/2. Cells were irradiated with 300 J/m^2 UVB, as described in Fig 1. Growth media without serum was added immediately, and cells were incubated at 37°. At the indicated time after irradiation, media was then removed and replaced with assay buffer containing [^{125}I]EGF (50 pM). Binding assays were performed, as described in *Materials and Methods*. This experiment was performed on two occasions with similar results.

Inhibition occurred within the first 1/2 h postirradiation and lasted for at least 6 h.

As shown in Fig 2, EGF bound in control cells actually increased from 0 to 4 h incubation time, then decreased. Binding in irradiated cells remained at a constant level. The change in [^{125}I]EGF binding during the 6-h incubation seen in control cells is most likely due to serum deprivation (after sham-irradiation cells were changed from growth media to assay buffer containing albumin). This pattern was seen consistently in replicate experiments. It should also be noted that no recovery from UV-induced inhibition was seen for at least 6 h postirradiation.

The effect of temperature on the UVB-induced inhibition of EGF binding was also tested in C3H/10T1/2 cells. Cells were irradiated or sham-irradiated at room temperature (23°C) or at 4°C, and EGF binding was determined at either 37°C or 4°C. Bound EGF was assayed at equilibrium for both temperatures: that is, at 37°C cells were incubated with ligand for 50 min, and at 4°C ligand binding was allowed to continue for 4 h [16]. As can be seen in Table I, UVB-induced inhibition of EGF binding was markedly temperature dependent. Inhibition of 71% occurred with irradiation (300 J/m^2) at either room temperature or 4°C, provided the binding assay was carried out at 37°C. No inhibition occurred when binding assay was performed at 4°C, regardless of temperature of irradiation procedure.

Because receptor internalization and degradation proceeds normally at 37°C, but not 4°C, we examined the possibility that UV radiation decreased EGF binding by simply increasing the internalization process. A comparison of cell-surface-associated [^{125}I]EGF and internalized ligand [19] determined by acid-wash experiments demonstrated that decreased binding of [^{125}I]EGF to irradiated cells was due to a proportionate decrease of [^{125}I]EGF in both compartments. In cells incubated at 37°C, the ratio of cell surface to internalized ligand was approximately 0.44 in both control and irradiated groups.

To characterize the nature of UVB-induced inhibition of EGF binding by Scatchard analysis, cells were irradiated or sham-irradiated and incubated with concentrations of [^{125}I]EGF from 0.5 to 150 ng/ml. EGF binding for Scatchard analysis was performed at 37°C rather than 4°C because inhibition does not occur when cells are maintained at 4°C immediately after irradiation. As shown in Fig. 3A, saturation of binding was achieved at slightly less than 50 ng/ml EGF, and maximal binding in UVB-treated cells was approximately 60% of that in control cells. Analysis of the Scatchard plot obtained from this data suggested resolution of a curvilinear plot into two binding sites (Fig 3B). Untreated cells contained high affinity sites with 4.3×10^3 sites per cell and a k_D of 0.2 nM, and low affinity sites with 4.7×10^4 sites per cell and a k_D of 33 nM. UVB treatment decreased the number of low affinity sites to 2.7×10^4 sites per cell without altering the affinity, and decreased the affinity of the high affinity sites to 0.8 nM without affecting the number of those sites.

Table I. Effect of Temperature on UVB-Induced Inhibition of ^{125}I-EGF Binding

Irradiation Temperature (Degrees)	Incubation Temperature (Degrees)	Percent Inhibition
23	37	71
23	4	0
4	37	71
4	4	0

C3H/10T1/2 cells were irradiated with 300 J/m^2 UVB or sham irradiated at the temperatures indicated above, as described in *Materials and Methods*. ^{125}I-EGF binding was initiated at the indicated temperatures immediately after irradiation and continued for 50 min at 37° or 4 h at 4°, and specific binding was determined as described in *Materials and Methods*. "Percent Inhibition" represents the decrease in specific binding measured in irradiated cells versus sham-irradiated cells. The experiment was repeated in its entirety with identical percent inhibition values.

UVB Radiation Effects on PKC The tumor promoting chemical 12-0-tetradecanoylphorbol-13-acetate (TPA) has been shown to

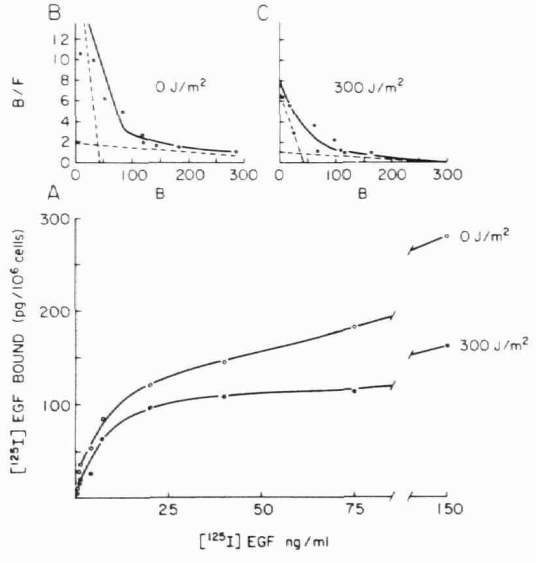

Figure 3. The effect of [^{125}I]EGF concentration on the specific EGF binding to C3H/10T1/2 cells treated with 0 or 300 J/m^2 of UVB. Irradiation and binding assays were performed as described in Fig 1 except that the concentration of [^{125}I]EGF varied between 0.5 and 150 ng/ml. Binding assays were performed at 37°C for 50 min. In Fig 3*A*, data are presented as mean specific [^{125}I]EGF bound for duplicate determinations. Scatchard analysis of data from this experiment using sham-treated and -irradiated cells are presented in Figs 3*B* and 3*C*, respectively. Data are presented in terms of [^{125}I]EGF bound (B) expressed as pg/10^6 cells versus Bound/Free (B/F) expressed in liters X 1.25×10^{-6}.

Table II. The Effect of UVB and TPA Treatment on Specific Ligand Binding to Cells in Culture

	EGF Binding (% of Control[a])	PDBU Binding (% of Control)
Treatment		
UVB (J/m²)[b]		
0	100	100
100	—	93
300	25	93
600	30	—
1000	—	105
TPA (ng/ml)[c]		
0	100	100
10	42	23
100	0	19

[a] Data is expressed as percent of control specific binding. Specific binding for both ligands was determined as described in *Materials and Methods*.

[b] Cells were exposed to UVB immediately before binding assay was begun, as in Fig 1.

[c] Cells were treated with TPA for 45 min before binding assay was begun as in *Materials and Methods*.

induce inflammation, hyperplasia and inhibition of binding of EGF and the phorbol ester PDBu to their membrane receptors [20–24]. TPA-induced inhibition of binding has been shown to result from the activation of protein kinase C (PKC), a Ca^{+2} and phospholipid-dependent kinase which catalyzes the phosphorylation of proteins at serine and threonine residues. TPA activation of PKC is associated experimentally with the translocation of enzyme activity from the cytosol to the membrane compartment [25]. PKC phosphorylates the EGF receptor at a specific threonine residue, resulting in decreased binding of ligand [26–28].

We performed further studies to determine whether TPA and UVB radiation share the same mechanism of action for inhibition of EGF binding. Experiments were designed to compare the effect of UVB radiation to TPA with regard to phorbol dibutyrate (PDBu) binding and PKC translocation. We first demonstrated that exposure to TPA (10 or 100 ng/ml) for 45 min decreases PDBu and EGF binding in C3H/10T1/2 cells (Table II). In the present study, UVB radiation (100, 300, and 1000 J/m²) of C3H/10T1/2 cells had no effect on PDBu binding (Table II). Cellular distribution of PKC was determined for C3H/10T1/2 cells treated with TPA, vehicle control (DMSO), UVB, and sham irradiation (Table III). Incubation with TPA caused a decrease in cytosolic PKC activity and a corresponding increase in membrane-associated PKC activity. When cells were harvested at one h postirradiation (150, 300, 600 and 1200 J/m²) and assayed for PKC activity, there was no difference in cytosolic and membrane-associated PKC between irradiated and control cells. In other experiments, 300 J/m² UVB failed to affect PKC activity at time points of 5 min, 1 and 2 h after irradiation (data not shown).

Table III. UVB-irradiation and TPA Effects on Protein Kinase C Activity in $C_3H/10T1/2$ Cells

UVB Dose J/m²	Cytosol	Membrane
0 (Sham)	2.3[a]	2.3
150	2.0	3.0
300	1.9	3.0
600	2.4	3.3
1200	2.7	3.3
TPA Dose		
0 (DMSO, 1 ul/ml)	5.0	3.5
250 ng/ml	0.3	17.0

Cells were harvested 1 h postirradiation, and cellular distribution of PKC activity was determined in C3H/10T1/2 cells, as described in *Materials and Methods*. The effect of TPA pretreatment was assayed in a separate experiment in which TPA or DMSO was added to growth media for 45 min at 37°C before cells were harvested.

[a] Data are expressed as p moles [^{32}P] transferred per ug protein per 10 min and represent the average of triplicate determinations per sample, which varied less than 10%.

DISCUSSION

Ultraviolet radiation is an agent known to induce several important responses in human and animal skin, including pigmentation, inflammation, induction of non-melanoma skin tumor growth, and hyperproliferation of epidermal keratinocytes [11,29,30]. The proliferation response is characterized by an initial inhibition of DNA, RNA and protein synthesis, followed within 24 h by stimulation of macromolecular synthesis [3]. The mechanism of this UV-induced proliferation stimulus has not been determined.

Other agents known to induce cellular proliferation act through second messenger systems in which protein kinases are activated, producing a cascade of signals. Such agents include the phorbol ester tumor promoters, polypeptide growth factors, and hormones. An early membrane event observed with many of these proliferative agents is the inhibition of epidermal growth-factor binding. TPA, in particular, shares a number of cellular effects with ultraviolet radiation, including promotion of non-melanoma skin-tumor growth, inflammation, and proliferation mediated by metabolites of arachidonic acid [21,23,31,32]. The mechanisms of cellular response to TPA have been characterized in much more detail than have those to UVB. Briefly, TPA binds to its membrane receptor, protein kinase C, which phosphorylates the EGF receptor (among other substrates), and inhibits EGF binding. It has been suggested that EGF receptor phosphorylation by TPA may play a role in the action of tumor promoters to potentiate the mitogenic effects of EGF [28]. Because both UVB and TPA induce epidermal hyperplasia, we were interested in examining the possibility that UVB acts on the same membrane-associated kinase systems; specifically, the EGF receptor and the phorbol ester receptor (protein kinase C).

The data presented here indicate that ultraviolet light in the range of 290–320 nm (UVB) does share with TPA a membrane-related effect on mammalian cells in culture: the inhibition of epidermal growth factor binding to cell-surface receptors. This effect is characterized by a curvilinear dose response and a decrease in the number of low affinity binding sites and a decrease in the affinity, but not number, of high affinity sites. It should be noted that studies for Scatchard analysis were performed at 37°C rather than 4°C. The lower temperature is standard, since it prevents internalization and degradation of receptors. Since our temperature studies indicated no inhibition of binding at the lower temperature, the Scatchard analysis was, by necessity, performed on cells at 37°C. Acid-wash experiments revealed that UV did not alter the internalization process, suggesting that the results of Scatchard analysis were likely a valid observation of UV-induced alteration of binding site kinetics.

The latter alteration is similar to that reported in Scatchard analysis of this cell type treated with TPA, suggesting at least partial similarity of method of action between UVB and TPA. Inhibition occurred at a very low exposure level of UVB (less than 150 J/m²), a dose which is within physiologic range and could be obtained in skin within min of natural summer-sun exposure. The UVB-induced inhibition of EGF binding was temperature dependent, as is TPA and bombesin-induced inhibition [33]. The temperature-sensitive step in the present study occurs after the initial UV-induced photochemical interaction, since inhibition occurred regardless of irradiation temperature. The postirradiation temperature was critical, with no inhibition occurring if the cells were incubated at 4°C. This suggested a mechanism involving a biologic process requiring enzyme mediation.

Unlike the effect of TPA treatment, UVB radiation-induced inhibition of EGF binding did not appear to occur through the activation and translocation of PKC. First, UVB exposure resulted in a down regulation of EGF binding, but had no effect on PDBu binding. In contrast, TPA treatment also induced down regulation of PDBu binding, modulating its own receptor. Second, UVB radia-

tion had no effect on PKC translocation when cells were assayed at time points from 5 min to 2 h after irradiation.

UVB inhibition of EGF binding may occur via several alternative mechanisms. The membrane lipid microenvironment of the EGF receptor may be perturbed by UVB photon induction of photoperoxidation [34,35]. However, the specificity of receptor binding inhibition would argue against such a generalized membrane disruption effect unless the EGF receptor had a chromophore, such as a tryptophan or cysteine residue, in a site critical for ligand binding. Alternately, UVB could stimulate autophosphorylation of the EGF receptor or phosphorylation by a kinase other than protein kinase C. Total kinase activity (non-Ca^{+2}, lipid and TPA dependent), however, did not increase as assayed in these studies. We have reported previously that UVB stimulates phospholipase activation and prostaglandin production in $C^3H/10T1/2$ cells [36], and others have reported UV induction of alterations in the cAMP system [37]. It may be that these closely related membrane signaling systems play a role in altering EGF binding.

Additional studies to define the mechanism by which UV induces alteration of EGF binding will lend insight into the effect of such radiation on membrane events, events which will likely prove important in UV control of cell growth and carcinogenesis.

We have also found that long-wave ultraviolet radiation inhibits EGF binding to this cell type, but with different effects on PKC activity [38].

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REGIONAL SKIN SYMPOSIUM IN COPENHAGEN

The International Society for Bioengineering and the Skin will present a Regional Symposium entitled "Rationales Behind Moisturizers in Dermatology," in the Rigshospitalet, Copenhagen, on Thursday, June 15th, 1989 (following the 25th Scandinavian Dermatology Meeting June 11–14th). For further details, please write to Jørgen Serup, M.D., Ph.D., and Tove Agner, M.D., Rigshospitalet, University of Copenhagen, Department of Dermatology H 5132, 9 Blegdamsvej, 2100 Copenhagen, Denmark.