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Relationship between Biological Responsiveness to Phorbol Esters and Receptor Levels in GH_4C_1 Rat Pituitary Cells

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ABSTRACT

During a 24-hr incubation of GH_4C_1 cells with phorbol esters or thyrotropin-releasing hormone, there is a decrease in the number of phorbol ester receptors (down-modulation). The purpose of this study was to investigate whether this decrease in receptor number attenuated cellular responsiveness to subsequent challenge with phorbol esters. Accordingly, cellular sensitivity to a phorbol ester-mediated biological response, namely the decrease in binding of epidermal growth factor, was compared in control and down-modulated cells. This phorbol ester-mediated event is closely associated with phorbol ester receptor occupancy, and it is therefore an effect for which altered dose-response characteristics correlating with alterations in the number of phorbol ester receptors could be anticipated. In fact, during a 48-hr exposure to phorbol esters, GH_4C_1 cells become refractory to the effect on epidermal growth factor binding. This time course is similar to that for the loss of phorbol ester receptors. However, when cells are down modulated by pretreatment with phorbol ester or thyrotropin-releasing hormone and then (re)challenged with phorbol ester, no differences in dose-response characteristics were observed between control and down-modulated cells. We therefore conclude that phorbol ester receptor down-modulation does not affect cellular responsiveness to phorbol esters, at least when decreased epidermal growth factor binding is used as the marker for the phorbol ester-mediated event.

INTRODUCTION

Phorbol esters elicit a variety of biological responses in many different cells and tissues (for reviews, see Refs. 1, 2, and 6). Most notably, phorbol esters are tumor promoters in mouse skin. In contrast to the abundance of information on cellular responses to phorbol esters, little is known about their mechanism of action. As a step in studying the mechanism of action, a technique for measuring the cellular binding of phorbol esters was developed by Driedger and Blumberg (8). With this assay, the binding affinities of several phorbol esters to homogenates of chick embryo fibroblasts and mouse skin were determined. The affinities agreed closely with the biological potencies of each ligand (5, 8). Thus, binding of phorbol esters to this receptor appears to be the initial event in mediating phorbol ester action on target tissues.

We have extended the original characterization of the binding to intact, living cells, thus permitting a closer comparison of binding parameters and biological responses (10). In particular, we have studied the characteristics of phorbol ester binding to GH_4C_1 cells (10), a continuous strain of rat pituitary cells (21). The biological responses of GH_4C_1 cells to phorbol esters can be divided into 2 categories. (*a*) Phorbol esters cause rapid responses related to membrane alterations, such as increased release of stored prolactin and decreased binding of 3 regulatory peptides (TRH,[4] somatostatin, and EGF) to their cell surface receptors (16). (*b*) Phorbol esters mediate long-term responses related to changes in biosynthetic activity, such as increased synthesis of prolactin and decreased production of growth hormone (17). The binding affinities for several phorbol esters and analogs correlate with their potencies in eliciting these biological responses (10).

There are now several reports of refractory responses to phorbol esters. With prolonged exposure to phorbol esters, 3T3 fibroblasts and B-16 melanoma cells "escape" from the phorbol ester-mediated inhibition of differentiation (7, 15). Similarly, the phorbol ester-mediated decrease in EGF binding is diminished with prolonged exposure to phorbol ester (13). Most intriguing is the report that hamster skin becomes refractory to the phorbol ester-mediated hyperplastic response, unlike mouse skin (20). Hamsters also are not sensitive to initiator-promoter-induced chemical carcinogenesis, unlike mice (20). These observations suggest that there are mechanisms by which cells can escape from phorbol ester-mediated biological effects, possibly including tumor promotion. Therefore, it is of interest to understand the molecular basis for these refractory responses.

In certain instances, modulation of target cell sensitivity to regulatory effectors is mediated through changes in either the number or affinity of specific cellular receptors (for review, see Ref. 4). We have described recently down-modulation of phorbol ester receptors in GH_4C_1 cells by both homologous ligands (phorbol esters and analogs) and the heterologous ligand, TRH (10). Therefore, it was of particular interest to examine whether down-modulation of phorbol ester receptors resulted in decreased cellular sensitivity to a receptor-mediated event. For this purpose, we have studied the phorbol ester-mediated decrease in EGF binding, a response which is closely linked to occupancy of the phorbol ester receptor and to which the cells demonstrate a refractory response. The results indicate that receptor down-modulation does not mediate this refractory response.

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[4] The abbreviations used are: TRH, thyrotropin-releasing hormone; EGF, epidermal growth factor; F10*, Ham's Medium F-10 supplemented with 15% horse serum and 2.5% fetal bovine serum; PDBU, phorbol 12,13-dibutyrate; ED_{50}, half-maximally effective dose.

MATERIALS AND METHODS

Growth of GH_4C_1 Cells. GH_4C_1 cells were grown in $F10^+$ (Grand Island Biological Co., Grand Island, N. Y.) as described (21). Cells were grown in 16-mm multiwell dishes (Falcon Plastics, Oxnard, Calif.) for 1 to 2 weeks, at which time they reached a plateau density of approximately 7×10^5 cells/well.

PDBU Binding Assay. Total PDBU binding was determined by incubating cells for 30 min at 37° with [^{3}H]PDBU (3.42 Ci/mmol) as described (10). Nonspecific binding was determined in the presence of 20 μM nonradioactive PDBU. The monolayers were then rinsed 3 times with 0.2 ml of $F10^+$ (4°). Cell sheets were dissolved in 0.1 N NaOH and prepared for liquid scintillation counting in Aquasol (New England Nuclear, Boston, Mass.). Specific binding was determined as total minus nonspecific cell-associated radioactivity.

In certain experiments in which cells were pretreated with PDBU, it was necessary to remove the nonradioactive PDBU before the remaining binding could be measured with [^{3}H]PDBU. Greater than 90% of the PDBU was removed by rinsing the cultures twice with $F10^+$ at 37° for 10 min each time (10).

EGF Binding Assay. Total EGF binding was determined by incubating cells for 45 to 60 min at 37° with ^{125}I-labeled EGF (~1200 Ci/mmol). Nonspecific binding was determined in the presence of 33 nM nonradioactive EGF. Following the equilibrium period, the cultures were rinsed with $F10^+$ as described above.

TRH Binding Assay. Total TRH binding was determined by incubating cultures for 60 min with ^{3}H-labeled TRH (100 Ci/mmol; New England Nuclear) as described by Hinkle and Tashjian (9). Nonspecific binding was determined in the presence of 2×10^{-7} M nonradioactive TRH. Following the equilibration period, the cultures were rinsed with $F10^+$ and processed for liquid scintillation counting as described above.

Materials. [^{3}H]PDBU was prepared by the method of Kreibich and Hecker (12). EGF was iodinated according to the method of Carpenter and Cohen (3). Materials were obtained from the following sources: EGF, Collaborative Research, Inc. (Waltham, Mass.); phorbol 12-myristate 13-acetate and mezerein, Chemical Carcinogenesis (Eden Prairie, Minn.). All others were from Sigma Chemical Co. (St. Louis, Mo.).

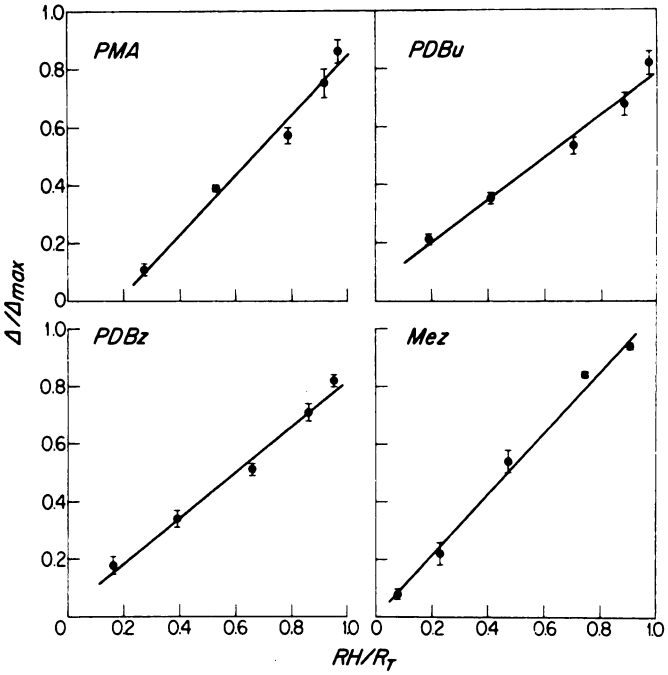

Chart 1. Structure-activity relationships for phorbol ester-mediated decrease in ^{125}I-labeled EGF binding. The loss of EGF receptors was determined in cultures treated for 5 min with increasing concentrations of the analogs followed by a 60-min incubation at 37° with ^{125}I-labeled EGF (30,000 cpm/well). The values for the decreases in EGF binding are the means ± range or S.E. determined in 2 or 3 separate experiments, respectively. Fractional receptor occupancy (RH/R_T) at each concentration of analog was calculated using K, values determined from dose-dependent inhibition of [^{3}H]PDBU binding. The validity of this approach was established by demonstrating that the binding follows a Michaelis-Menten function as indicated by the Hill coefficients of the displacement curves which are as follows: phorbol myristate acetate (*PMA*), 1.0 ± 0.1 (n = 3); PDBU, 0.85 ± 0.03 (n = 2); phorbol dibenzoate (*PDBz*), 0.87 ± 0.10 (n = 2); and mezerein (*Mez*), 1.00 ± 0.05 (n = 3). The K, values (nM) were as follows: phorbol myristate acetate, 4.3 ± 1.3 (n = 3); PDBU, 6.7 ± 0.1 (n = 3); phorbol dibenzoate, 23 ± 5 (n = 3); mezerein, 55 ± 6 (n = 3). Values are mean ± S.E. or range. Δ/Δ_{max} = fractional maximal obtained response.

RESULTS

Phorbol Ester Effects on EGF Binding Tightly Linked to Receptor Occupancy. In order to investigate whether loss of phorbol ester receptors resulted in a decreased sensitivity to a phorbol ester-mediated response, it was important to study a response which was tightly linked to receptor occupancy. The phorbol ester-mediated decrease in EGF binding fulfilled this requirement according to 3 criteria. (*a*) The relationship between fractional receptor occupancy and fractional maximal response was linear for each of 4 ligands over the range of 10 to 90% receptor occupancy (Chart 1). The slopes of the lines relating receptor occupancy to the biological response were 0.75 to 1.0 for each ligand. Consequently, 50% receptor occupancy resulted in approximately 50% of the maximal response. (*b*) A second property of the phorbol ester-mediated decrease in EGF binding which also indicated that it was a response tightly linked to occupancy of the phorbol ester receptor was the rapidity of the response. The effect was observed with less than a 5-min pretreatment with phorbol ester. (*c*) The phorbol ester effect was rapidly reversible. Removal of PDBU with a thorough washing procedure resulted in immediate recovery of EGF binding to control levels (see below). The difference between the fast recovery from PDBU treatment reported here and the lengthy recovery times reported by Shoyab *et al.* (19) for 3T3 cells following treatment with phorbol 12-myristate 13-acetate is probably due to incomplete removal of that substantially more lipophilic phorbol ester.

Under these conditions, in which a biological response is directly proportional to receptor occupancy, a decrease in the total number of receptors would be predicted to decrease the maximal extent of the response of the cells to the ligand, assuming no other relevant changes occurred. This is in contrast to the predicted shift in the dose-response curve for a biological response in which there was evidence of "spare receptors" (*i.e.*, the $ED_{50} < K_d$). In this case, a decrease in receptor number should result in an increase in the ED_{50} and a decrease in the maximal extent of the response only when the decrease in receptor number becomes a limiting event (4).

GH_4C_1 Cells Refractory to Phorbol Ester-mediated Decrease in EGF Binding but not TRH Binding. Exposure of cells to phorbol esters resulted in a concentration-dependent decrease in binding of EGF. The maximal effect was observed with a 5-min preincubation with phorbol ester followed by a 60-min incubation with ^{125}I-labeled EGF (in the continued presence of the phorbol ester), which is the optimal time for measuring EGF binding to its receptor in these cells (18). When the pretreatment period was increased to 7 or 23 hr, the effect of phorbol esters was diminished (Chart 2). This decrease in responsiveness occurred slowly as did the down-modulation of

phorbol ester receptors (Chart 2). Within the practical limits of the concentrations of phorbol esters used, a decrease in the maximal obtainable response was observed (86 ± 1% decrease *versus* 41 ± 1% decrease in EGF binding for 5-min and 23-hr pretreatment, respectively; mean ± S.E., n = 3). No marked increase in the apparent ED_{50}s for 5-min *versus* 23-hr pretreatment was noted. These observations correlate with the predicted effect of decreased number of receptors on a biological response which is a linear function of receptor occupancy (see above).

Down-modulation of phorbol ester receptors and the attenuated cellular responsiveness to the phorbol esters both exhibited temperature dependence; both were observed at 37° but not at 24° in an experiment of 24-hr duration (data not shown). Additional evidence for a role of down-modulation in the generation of the refractory response was observed in a human osteosarcoma cell line (SaOs-2). These cells did not become significantly refractory to the phorbol ester-mediated decrease in EGF binding, nor did they down modulate phorbol ester receptors over a 48-hr period (data not shown).

In contrast to the decreased phorbol ester effect on EGF binding during prolonged exposure to phorbol esters, the effect on TRH binding did not diminish (Table 1). The phorbol ester-mediated decrease in TRH binding is maximal following a 3-hr pretreatment with phorbol ester, followed by a 1-hr equilibration with ^{3}H-labeled TRH in the continued presence of phorbol ester (16). That is, this effect is slower than the effect on EGF binding. This may indicate that phorbol ester-mediated decrease in TRH binding is a response mediated by events more distal to the phorbol ester receptor interaction and thus may not be a sensitive indicator of the consequences of receptor down-modulation. On the other hand, the lack of refractoriness in the TRH receptor response may indicate that the refractoriness of the EGF receptor response is unique to the EGF receptor and is not related to phorbol ester receptor down-modulation.

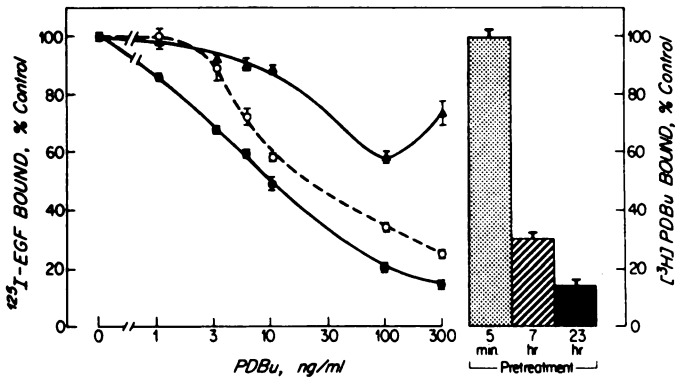

Chart 2. Decrease in phorbol ester-mediated response and phorbol ester binding with prolonged exposure to PDBU. GH_4C_1 cells were pretreated with increasing concentrations of PDBU for 5 min (●), 7 hr (○), or 23 hr (▲). ^{125}I-labeled EGF was then added for a 60-min equilibration period in the continued presence of PDBU. Nonspecific binding was determined in the presence of excess unlabeled EGF. The decrease in phorbol ester binding was determined in cultures pretreated for the times indicated with 100 ng PDBU per ml (a maximally effective concentration). PDBU was removed from the cultures by a thorough washing procedure (10), and the residual binding was measured with [^{3}H]PDBU. Similar results were obtained in 2 other experiments. EGF binding in the absence of PDBU was 999 ± 16, 1043 ± 1, and 1194 ± 95 cpm for 5-min, 7-hr, and 23-hr pretreatments with PDBU, respectively (mean ± range of duplicate determinations). PDBU binding in the absence of PDBU pretreatment was constant throughout the experiment (855 ± 31 cpm/well, 4.2 ± 0.2 pmol/mg cell protein).

Dose-Response Characteristics of Down-modulated Cells. When cells are treated with either TRH or phorbol esters for prolonged periods (>6 hr), there is a loss of [^{3}H]PDBU binding (10). This binding returns slowly following removal of TRH or PDBU (*i.e.*, there is no significant recovery within 2 hr). Therefore, it was possible to expose cells to TRH or PDBU for 18 to 24 hr, at which time [^{3}H]PDBU binding was maximally decreased, and then to remove the TRH or PDBU and compare the dose-response characteristics of control and down-modulated cells to subsequent challenge with PDBU. Following pretreatment with and removal of either TRH or PDBU, minor effects on the magnitude of EGF binding were observed [102 ± 7% of control (n = 9); and 116 ± 8% of control (n = 5); (mean ± S.E.) for TRH-pretreated and PDBU-pretreated cells, respectively).

The dose-response curves for the phorbol ester-mediated decrease in EGF binding in control and down-modulated cells are shown in Chart 3. No effect of down-modulation on decreasing the maximal extent of the response was observed. Likewise, only minor and variable effects on ED_{50} were observed. In 5 of 9 experiments with TRH down-modulated cells, no change in ED_{50} was observed. In the remaining 4 experiments, a 1.7- to 3-fold increase in the ED_{50} was observed which did not correlate quantitatively with the extent of loss of [^{3}H]PDBU binding. In 5 of 5 experiments with cells down modulated by pretreatment with PDBU, no significant decrease in sensitivity was observed.

Lack of Evidence for Receptors with Increased Potency in Down-modulated Cells. The lack of decreased responsiveness in cells which have fewer phorbol ester receptors could result from positive cooperativity among the remaining receptors. No

Table 1

Effect of phorbol ester treatment on ligand binding

GH_4C_1 cells were exposed to PDBU (100 ng/ml), and the effects on binding of [^{3}H]PDBU, ^{3}H-labeled TRH, and ^{125}I-labeled EGF were measured at the indicated times. Medium ± PDBU was replenished at 24-hr intervals. PDBU binding was measured with 10 nM [^{3}H]PDBU following removal of unlabeled PDBU by thorough washing. TRH binding was measured with 2.4 nM ^{3}H-labeled TRH. EGF binding was measured with 2×10^{-11} M ^{125}I-labeled EGF. The acute effects of PDBU on TRH or EGF binding were measured following a 3-hr or 5-min preincubation with PDBU, respectively, followed by addition of labeled peptide for a 1-hr incubation at 37°. These time intervals have been shown to be optimal for measuring the acute effects of phorbol esters on these biological responses (16). Similar results were obtained in 2 other experiments, one of which was continued for 6 days.

	[^{3}H]PDBU bound (pmol/mg)		^{3}H-TRH bound (pmol/mg)		^{125}I-EGF bound (fmol/mg)	
	Control	+ PDBU	Control	+ PDBU	Control	+ PDBU
Acute response	1.08 ± 0.01		0.21 ± 0.01	0.10 ± 0.01 (46%)	4.1 ± 1.0	0.59 ± 0.08 (12%)
24-hr treatment	1.31 ± 0.12	0.40 ± 0.01 (31%)[a]	0.48 ± 0.02	0.15 ± 0.01 (35%)	4.8 ± 0.41	1.9 ± 0.02 (40%)
90-hr treatment	1.27 ± 0.05	0.26 ± 0.01 (20%)	0.41 ± 0.01	0.14 ± 0.04 (35%)	4.6 ± 0.01	4.5 ± 0.04 (98%)

[a] Numbers in parentheses, percentage of control value.

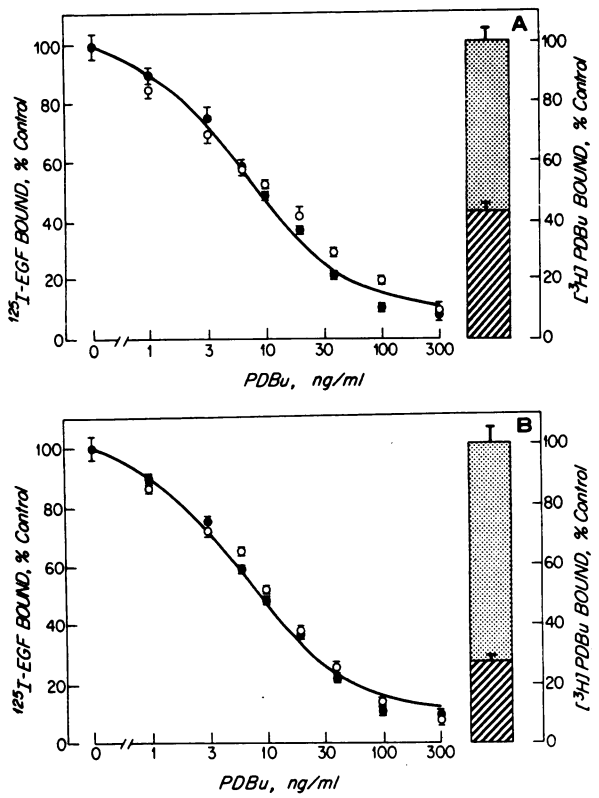

Chart 3. Cells were pretreated for 24 hr with 2×10^{-7} M TRH (*A*) or 10 ng PDBU per ml (*B*). Cultures were rinsed twice with serum-containing medium and then incubated at 37° for 1 hr in serum-containing medium to remove residual TRH or PDBU. Fresh medium was added, and the cells were returned to the incubator for an additional 2 hr. At the end of this pretreatment period, fresh medium was added, the cells were exposed to PDBU for 5 min, and then ^{125}I-labeled EGF was added for a 1-hr incubation at 37°. [^{3}H]PDBU binding was determined in parallel cultures. The *solid lines* are drawn according to the equation

$$\Delta = \frac{(\Delta_{max})(S)}{K_D + S}$$

where K_D is 11 nM, S is concentration of PDBU, and Δ_{max} is 90% decrease in EGF binding. ●, EGF binding in control cultures; ○, EGF binding in down-modulated cultures. *Stippled bars*, [^{3}H]PDBU binding in control cultures, *cross-hatched bars*, [^{3}H]PDBU binding in down-modulated cultures. Data are mean ± range of duplicate determinations.

evidence for cooperativity in binding to the phorbol ester receptor (see legend to Chart 1) or in eliciting the biological responses was observed; that is, the slopes of the Hill plots were approximately 1. (Hill coefficients for phorbol ester-mediated decrease in EGF binding were as follows: control, 1.12 ± 0.14 (n = 5); TRH pretreated, 0.79 ± 0.08 (n = 4); and PDBU pretreated, 1.0 ± 0.10 (n = 2); (mean ± S.D. or range.) Linear transformation of the dose-response data resulted in a single straight line. Thus, there was no consistent evidence for a second, more potent class of receptors in down-modulated cells. These results are consistent with the relatively good fit of the actual data to the theoretical dose-response curves drawn in Chart 3.

DISCUSSION

The phorbol ester-mediated decrease in EGF binding is a biological response which occurs rapidly and correlates well with occupancy of phorbol ester receptors. Therefore, it was a likely candidate for a response which may be altered by a change in the number of phorbol ester receptors. In fact, GH_4C_1 cells did become refractory to the phorbol ester-mediated decrease in EGF binding with continued exposure to phorbol ester over a period of 24 to 48 hr. This refractory response correlated with the loss of phorbol ester receptors with respect to time course and temperature dependence. In contrast, GH_4C_1 cells did not become refractory to the phorbol ester-mediated decrease in TRH binding.

The link between biological response and receptor occupancy was characterized further by taking advantage of the heterologous down-modulation of phorbol ester receptors by TRH, which in itself has an insignificant effect on EGF binding. No reproducible difference in dose-response characteristics between control and down-modulated cells was observed. No evidence for cooperativity or a subclass of highly effective receptors was found. Similarly, no differences in dose-response characteristics were observed with cells which were rechallenged with phorbol ester following homologous down-modulation and washout of the phorbol ester. This combination of results indicates that down-modulation of phorbol ester receptors does not explain the decreased responsiveness of the cells to phorbol esters, at least when the response measured was a decrease in EGF binding. It appears that the correlation between the escape observed with continued exposure to phorbol ester and the decreased number of receptors is coincidental.

Several categories of possibilities exist which may explain why the down-modulated cells do not demonstrate decreased sensitivity to phorbol esters. (*a*) Despite the close correlation between fractional receptor occupancy and fractional maximal response in control cells, the response measured (decreased binding of ^{125}I-labeled EGF) may be too distant from the receptor-ligand interaction to be an accurate indicator of a response which is regulated by the number of occupied receptors. Evidence that this response is not tightly coupled includes the facts that phorbol esters do not cause a decrease in EGF binding in cells treated at 4° or in isolated membrane preparations (13, 14, 19). (*b*) Down-modulated cells may become hypersensitive to the phorbol ester-mediated response due to other consequences of phorbol ester or TRH treatment. For example, alterations in the lipid composition of the membranes could result in changes in activity of enzymes or transport proteins which are involved in mediating the response. Thus, occupancy of the smaller total number of phorbol ester receptors in down-modulated cells could result in a biological response of the same magnitude as in control cells. (*c*) It is possible that several domains of phorbol ester receptors exist and that those receptors which are involved in the phorbol ester-mediated decrease in EGF binding are spared from down-modulation. Thus, even a severe defect in phorbol ester binding might result in selective loss in cellular responsiveness to that effector, while other cellular responses to phorbol esters would be maintained. An analogous explanation has been proposed to explain differential cellular responsiveness to insulin in certain cells with severe defects in insulin binding (11).

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