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Relationship between Mezerein-mediated Biological Responses and Phorbol Ester Receptor Occupancy

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ABSTRACT

The phorbol ester analog, mezerein, is a weak complete and Stage 1 tumor promoter; however, it is as potent as the most active phorbol esters as a second stage promoter and inflammatory agent. Therefore, mezerein is a useful compound for studying responses associated with Stage 1 or Stage 2 promotion. In this paper, we show that in G-292 osteosarcoma cells in culture, mezerein is 25-fold more potent in causing a decrease in binding of epidermal growth factor to its specific cellular receptor than in inducing prostaglandin E_2 production. This differential potency for these two actions was not noted for other phorbol esters. Our findings indicate that mezerein interacts with the major phorbol dibutyrate receptor to increase prostaglandin E_2 production and also either with a distinct cellular target with a higher affinity or the same target with increased efficacy to cause a decrease in the binding of epidermal growth factor. These human osteosarcoma cells thus provide a model system to facilitate analysis of phorbol ester receptor heterogeneity.

INTRODUCTION

Phorbol esters are potent tumor promoters and inflammatory agents in mouse skin. Within a series of phorbol esters and related diterpenes, the potencies of a compound as both a tumor promoter and an inflammatory agent are generally comparable. However, MEZ,[5] a nonphorbol ester diterpene, is comparable in efficacy to the most potent phorbol ester, PMA, as an inflammatory agent but is 50 times less potent as a complete tumor promoter (11); *i.e.*, following exposure to a subthreshold dose of an initiating carcinogen, twice-weekly treatment with PMA leads to tumor formation; MEZ is 50-fold less potent than is PMA with this protocol (complete tumor promotion). The process of tumor promotion in mouse skin has been divided into at least 2 stages (2, 10, 15). If mice are treated with PMA for 2 weeks (Stage 1), MEZ can substitute for PMA with nearly the same potency in completing the tumor formation process over the next several weeks (Stage 2). Therefore, MEZ is relatively inactive as a Stage 1 promoter; however, MEZ is nearly equipotent to PMA as a Stage 2 promoter. These results have led to the hypothesis that some MEZ responses are mediated via MEZ interaction with the phorbol ester receptor associated with Stage 1 promotion, but the possibility that different receptors also exist is left open to explain the much greater potency of MEZ as a Stage 2 promoter.

Specific cellular receptors for phorbol esters can be detected using [^{3}H]PDBU (6, 8). The binding affinities of phorbol esters and related compounds can be estimated by their potencies as competitors for [^{3}H]PDBU binding sites. In mouse skin homogenates, the rank order of affinities of several ligands for the PDBU receptor parallels the rank order of complete tumor-promoting potencies (5), indicating that the receptor that is being measured is related to the cellular target for promotion *in vivo*. MEZ can completely displace [^{3}H]PDBU from its binding sites, and the affinity of MEZ for the PDBU receptor reflects its potency as a complete or Stage 1 tumor promoter rather than as a Stage 2 promoter or an inflammatory agent. Therefore, it has been proposed that MEZ acts as a complete or Stage 1 tumor promoter via interaction with the major PDBU receptor but that a distinct MEZ-binding site may also exist to explain its potency as an inflammatory or Stage 2 promoting agent.

To date, the published data on PDBU binding have indicated only one class of high-affinity PDBU-binding sites in several cell types (8, 12, 17). The ED_{50}s for phorbol ester- and MEZ-mediated biological responses correspond closely to the apparent dissociation constants for binding to the PDBU receptor (8, 9). Therefore, the available evidence is consistent with the hypothesis that the receptor that is measured with [^{3}H]PDBU is the cellular target which mediates both phorbol ester- and MEZ-induced biological responses.

We now report evidence that is strongly indicative of a cellular target for MEZ that is distinct from the major PDBU receptor. Our evidence is based on a comparison of ED_{50}s for 2 MEZ-mediated biological responses compared to the apparent dissociation constants of MEZ for the phorbol ester receptor, as measured by competition for [^{3}H]PDBU binding sites. For these studies, we have used human osteosarcoma cells, which offer the advantage of responding to phorbol esters in 2 ways that (*a*) may be relevant to tumor promotion and (*b*) can both be measured after a brief exposure to phorbol esters or MEZ. The first response is the phorbol ester-mediated decrease in binding of EGF, a response which may be relevant to tumor promotion *in vivo* in that it suggests a means by which phorbol esters may interfere with the normal transduction of information received as a membrane signal. The second response is phorbol ester-stimulated production of PGE_2. These 2 responses are measured under the same conditions and over the same intervals of exposure to agonist, thus ruling out the trivial possibility that differential potencies are related to differ-

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[5] The abbreviations used are: MEZ, mezerein; PMA, phorbol myristate acetate; PDBU, phorbol dibutyrate; ED_{50}, 50% effective dose; PGE_2, prostaglandin E_2; EGF, epidermal growth factor; F10$^+$, Ham's F10 medium supplemented with 15% horse serum and 2.5% fetal bovine serum.

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ential availability or catabolism of the ligands. Such a problem has been a confounding difficulty in comparisons of potencies of compounds as inflammatory *versus* tumor-promoting agents (1).

MATERIALS AND METHODS

Cell Culture. G-292 cells (CRL 1423; American Type Cell Culture Collection, Rockville, Md.) were grown to confluence in 24-well dishes (Falcon) in $F10^+$ (13).

[^{3}H]PDBU Binding Assay. For binding analysis, the cells were incubated in 200 μl $F10^+$, 50 μl [^{3}H]PDBU (20 Ci/mmol), and 50 μl $F10^+$ containing either 0.75% dimethyl sulfoxide or 0.75% dimethyl sulfoxide plus PDBU (final concentration, 20 μM). After equilibration at 37° for 30 min, an aliquot of the supernatant was processed for liquid scintillation counting to determine the concentration of free [^{3}H]PDBU. The cell monolayers were then washed quickly 3 times with 0.2 ml of ice-cold $F10^+$, dissolved, and processed for liquid scintillation counting (8). Specific binding was calculated from the difference in cell-associated radioactivity measured in the absence or presence of PDBU. Nonspecific binding was a linear function of the concentration of free [^{3}H]PDBU.

^{125}I-EGF Binding Assay. Fresh $F10^+$ medium without or with phorbol-related compounds and ^{125}I-EGF (80,000 cpm, 2.6×10^{-11} M) in 0.5 ml were incubated with G-292 cells for 90 min at 37°. Previous studies of the time course of association of EGF with these cells have shown that a 90-min incubation results in maximal binding (14). After equilibration, the monolayers were washed as described above, dissolved in 0.1 N NaOH, and processed for counting of γ-radiation. Nonspecific binding was determined in the presence of 10^{-8} M EGF.

PGE_2 Radioimmunoassay. PGE_2 was measured by radioimmunoassay using specific antisera, a generous gift of Dr. L. Levine (Brandeis University, Waltham, Mass.) as described (7).

Materials. [^{3}H]PDBU (20 Ci/mmol) and [^{3}H]PGE_2 (180 Ci/mmol) were from New England Nuclear (Boston, Mass.). Mouse EGF was prepared in our laboratory and iodinated by the method of Carpenter and Cohen (3) to a specific activity of 1520 Ci/mmol. PDBU was from Sigma Chemical Co. (St. Louis, Mo.), and PMA and MEZ were from Chemical Carcinogenesis (Eden Prairie, Minn.).

RESULTS

Scatchard analysis of the binding of [^{3}H]PDBU to confluent monolayers of human osteosarcoma cells is shown in Chart 1. In 4 experiments, Scatchard analysis in the concentration range of 2 to 50 nM showed evidence of only one binding site with K_d = 12 ± 3 nM (mean ± S.D.; $r^2 > 0.99$ in each experiment). At concentrations greater than 50 nM, data points did not always fall on the line described by the lower concentrations. We did not characterize this further because: (*a*) at the higher concentrations, nonspecific binding is greater than 60% of the total binding, thus making these data relatively less reliable; and (*b*) the ED_{50}s for the biological responses to PDBU correspond closely to the K_d for the receptor characterized by the low concentrations. If the discrepancy with the higher concentrations was indicative of a second binding site, it would be of low affinity (K_d > 100 nM) for which the biological response data do not correlate.

The affinity of the receptor for PMA and MEZ was estimated by the potency of these compounds as competitors for [^{3}H]-PDBU binding (Chart 2). PMA was highly potent with a K_i = 2 nM; MEZ was a poor competitor with a K_i > 200 nM. Similar competition curves for MEZ binding to the PDBU receptor were

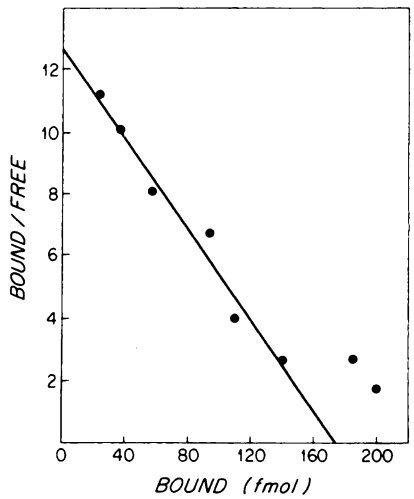

Chart 1. Specific binding of [^{3}H]PDBU to G-292 cells. G-292 cells were incubated with increasing concentrations of [^{3}H]PDBU. Specific binding was calculated from the difference in cell-associated radioactivity measured in the absence and presence of excess nonradioactive PDBU at each concentration of [^{3}H]PDBU. Nonspecific binding was a linear function of the concentration of free [^{3}H]PDBU. The data were analyzed according to the method of Scatchard (13), and the line is drawn according to linear regression analysis. Each *point* was determined in duplicate. The data shown are mean values, and deviations from the mean were less than ±10%.

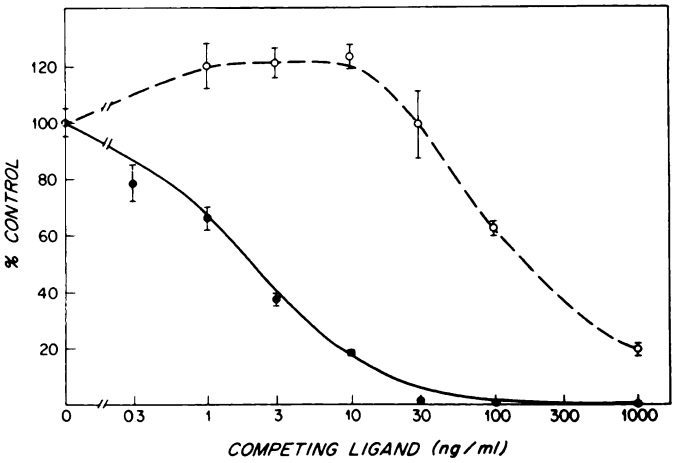

Chart 2. Competition by PMA and MEZ for [^{3}H]PDBU binding to G-292 cells. Binding was measured as described in "Materials and Methods" in the presence of the indicated concentrations of PMA (●) and MEZ (○). The apparent dissociation constants (K_is for competing ligands) were calculated according to the method of Cheng and Prusoff (4) using 2 nM [^{3}H]PDBU, a concentration which occupies about 15% of the available PDBU-binding sites. Similar results for competition by MEZ were obtained by using 35 nM [^{3}H]PDBU, a saturating concentration. *Points*, mean of 3 dashes; *bars*, S.D. *Ordinate*, percentage of control specific [^{3}H]PDBU binding in the absence of competing ligand. The purity of both compounds was confirmed by high-performance liquid chromatography.

obtained with either saturating or subsaturating concentrations of [^{3}H]PDBU.

Dose-response curves for PMA, PDBU, and MEZ for the decrease in ^{125}I-EGF binding and increase in PGE_2 production are shown in Chart 3. For PMA (Chart 3, *top*), similar ED_{50}s for the 2 responses were noted (3.2 ± 1.6 nM for the decrease in EGF binding and 6.7 ± 2.6 nM for the increase in PGE_2 production). These were both within a factor of 3 of the K_i for PMA binding. Similar results were obtained for PDBU (Chart 3, *middle*). The ED_{50}s for the effect on EGF binding were 13 ± 4.0 and 21 ± 3.2 nM for PGE_2 production. Again, both were within a factor of 2 of the K_d. Strikingly different results were

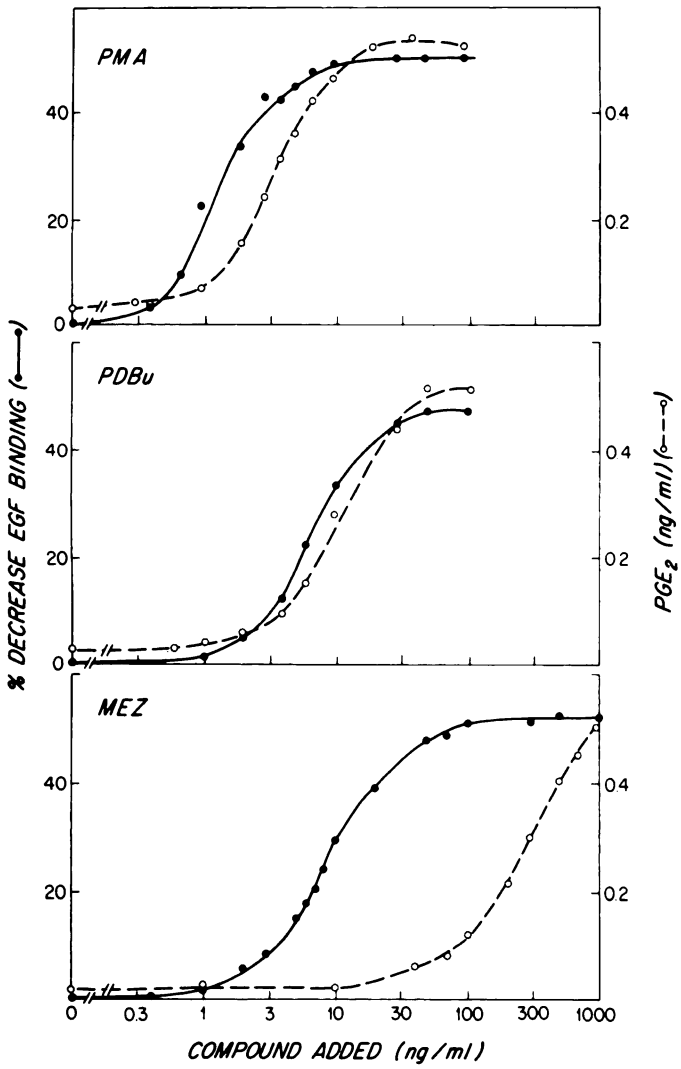

Chart 3. Increased PGE_2 production and decreased ^{125}I-EGF binding to G-292 osteosarcoma cells with increasing concentrations of 3 phorbol-related compounds. Fresh F10$^+$ medium (0.5 ml) with the indicated concentrations of either PMA, PDBU, or MEZ was added, and specific ^{125}I-EGF binding and PGE_2 production were measured in 90-min incubations at 37° (14). Data are expressed as the decrease in ^{125}I-EGF binding as compared to untreated cultures in which specific binding was measured as 6175 ± 205 cpm. PGE_2 from triplicate cultures was measured by radioimmunoassay, and standard errors were less than 5% of the mean value. For each treatment group, there were 2.0 ± 0.1 μg cellular DNA per dish.

obtained with MEZ (Chart 3, *bottom*). In this case, the ED_{50}s for the 2 responses (12.8 ± 3.2 nM for the effect on EGF binding and 320 ± 112 nM for PGE_2 production) differed by about 25-fold. These experiments were repeated 6 times; in each experiment, the same large difference was seen for the 2 responses to MEZ. It is the ED_{50} for the action of MEZ on PGE_2 production which agrees most closely with the K_i for MEZ binding to the PDBU receptor.

We have eliminated trivial explanations for the large difference in ED_{50}s for the MEZ-mediated responses. (*a*) Binding analyses and biological responses were measured in the same medium and over the same time period to make the closest possible comparison between dissociation constants and both ED_{50}s. (*b*) Purity of the MEZ was confirmed by high-performance liquid chromatography, thus ruling out the possibility that the differences in ED_{50}s were due to an impurity in the preparation. (c) Finally, if medium containing MEZ was incubated with cells for 90 min and then transferred to fresh cells for an ^{125}I-EGF-binding experiment, the same ED_{50} for the decrease in ^{125}I-EGF-binding (12.8 nM) was found as if fresh medium containing MEZ was used (12.9 nM). Thus, there appears to be no appreciable metabolism of MEZ to biologically inactive compounds during the incubation period used.

DISCUSSION

It has been recognized that certain phorbol esters have differential potencies as inflammatory *versus* tumor-promoting agents. These include 12-deoxyphorbol derivatives with short-chain substituents and unsaturated fatty acid derivatives of phorbol (1). We tested whether unsaturation of the C-12 and C-13 substituents might be important in determining binding specificity for either the MEZ- or PDBU-binding sites by comparing the potencies of phorbol dihexanoate and phorbol dihexadienoate in eliciting the 2 biological responses. Both compounds were complete agonists for each response, and all ED_{50}s were about 10 ng/ml. Therefore, unsaturation at C-12 and C-13 does not appear to be important in distinguishing between the MEZ- and the PDBU-binding sites in G-292 cells.

Our findings are significant because they demonstrate clearly a large difference between the K_i for MEZ for binding to the PDBU receptor and the ED_{50} for causing a biological response. In GH_4C_1 rat pituitary cells, the MEZ-mediated decrease in EGF binding is a linear function of MEZ occupancy of the PDBU receptor (9). Also, the ED_{50} for MEZ-mediated down modulation of the PDBU receptor is approximately equal to its K_i (8). In D16 mouse pituitary corticotrophs, the half-maximal response for stimulation of adrenocorticotropic hormone release by MEZ is approximately equal to its K_i for the PDBU receptor.[6] Therefore, the osteosarcoma cells can be utilized as a model for analysis of a response to MEZ which does not appear to be mediated through its interaction with the major PDBU receptor. We should point out that the slight increase in binding observed with low concentrations of MEZ (Chart 2) was reproducible, although we do not understand the significance of this observation at the present time.

We interpret our findings to indicate that MEZ interacts with the PDBU receptor to increase PGE_2 production, presumably via activation of a cellular phospholipase activity. In addition, we suggest that these data imply that MEZ also interacts with a distinct cellular target with high affinity to cause a decrease in binding of EGF. This target may or may not be a subset of the total PDBU receptor population. An alternative explanation would be that MEZ interacts with only one target but demonstrates differential efficacy in eliciting the 2 biological responses. This would require a unique interaction of MEZ with the receptor because the differential potency was not noted for PDBU and PMA. Differential potencies of pharmacological agents are most often explained by receptor heterogeneity; however, resolution of the question of heterogeneity *versus* differential efficacy will require development of a suitable radiolabeled ligand for direct measurement of the MEZ receptor. Although MEZ itself is probably too lipophilic to serve as the labeled probe, the osteosarcoma cell system that we have

[6] M. Phillips and S. Jaken, submitted for publication.

described should prove useful in identifying suitable structural analogs by comparing their ED_{50}s for the 2 different biological responses.

With this unique human cell culture system, other biological consequences of occupancy of either the PDBU or the MEZ receptor can now be explored. Inhibition of responses related to specific occupancy of PDBU or MEZ receptors can be evaluated by using compounds which have been reported to be specific inhibitors of either Stage 1 or Stage 2 promotion (16). These results would help to clarify the relationship between the proposed receptor classes in G-292 cells and those in mouse skin related to Stage 1 or Stage 2 promotion.

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