Identification of receptors for phorbol ester tumor promoters in intact mammalian cells and of an inhibitor of receptor binding in biologic fluids

(carcinogenesis/phorbol dibutyrate/serum factors/teleocidin)

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ABSTRACT Utilizing [^3H]phorbol dibutyrate [$\text{P}(\text{Bu})_2$], we have developed an assay for high-affinity phorbol ester receptors in intact rat embryo fibroblasts. At 37°C , binding of [^3H]P(Bu) $_2$ reached a maximum within 10 min and was rapidly reversible. The tumor promoters 12-O-tetradecanoyl-phorbol 13-acetate, teleocidin B, and mezerein were potent inhibitors of [^3H]P(Bu) $_2$ binding. Phorbol and 4- α -phorbol didecanoate, which lack tumor-promoting activity, did not inhibit [^3H]P(Bu) $_2$ binding. Epidermal growth factor, platelet-derived growth factor, fibroblast growth factor, arginine and lysine vasopressin, luteinizing-hormone releasing hormone, and diazepam did not inhibit [^3H]P(Bu) $_2$ binding. A Scatchard analysis was compatible with two classes of binding sites, one with $K_d = 8 \text{ nM}$ and about $1-2 \times 10^5$ sites per cell and the other with $K_d = 710 \text{ nM}$ and about 3×10^6 sites per cell. Sera from various species, human amniotic fluid, and certain tissue extracts inhibited specific binding of [^3H]P(Bu) $_2$. Fractionation of human serum led to a 135-fold purification of an inhibitory factor with a molecular weight in the range 40,000 to 80,000.

In contrast to chemicals that initiate the carcinogenic process apparently by binding covalently to cellular DNA, the primary site of action of the phorbol ester tumor promoters appears to be the cell surface membrane (for review, see refs. 1 and 2). In previous papers (3, 4) from this laboratory, it was postulated that the potent tumor promoter 12-O-tetradecanoylphorbol 13-acetate (TPA) actually binds to and usurps the function of a membrane-associated receptor that is normally utilized by an endogenous polypeptide growth factor. Indirect evidence for this hypothesis includes the facts that: (a) TPA and related compounds act in a concentration range similar to that of several hormones and growth factors (i.e., 0.1–10 nM), (b) these compounds display similar structure–function requirements on cells from diverse species and tissues, and (c) like known hormones, they induce highly pleiotropic effects which vary considerably depending upon the target cell (3, 4). The fact that the earliest cellular responses to these agents occur at the cell surface membrane (1, 2) suggested that the putative receptors were associated with the plasma membrane (2–4).

Utilizing [^3H]TPA, however, we were not able to demonstrate the existence of a specific saturable receptor in intact HeLa cells but we were concerned that these receptors might have been masked by the nonspecific partitioning of the highly hydrophobic TPA into lipid phases of the cell (5). Recently, Blumberg and his colleagues utilized [^3H]phorbol dibutyrate [P(Bu) $_2$], which is much less hydrophobic, to overcome this problem and obtained direct evidence of specific high-affinity

saturable receptors in crude membrane preparations of chicken embryo fibroblasts (6) and mouse epidermis (7).

In the present study, we utilized [^3H]P(Bu) $_2$ to demonstrate cell surface receptors for this class of compounds in intact cell monolayers of rat embryo fibroblast cultures. This paper describes several properties of these receptors and also provides evidence that mammalian sera and tissue extracts contain a factor that inhibits [^3H]P(Bu) $_2$ binding.

MATERIALS AND METHODS

Materials. The rat embryo cells (CREF) used are a subclone of the Fischer cell line, isolated by Paul Fisher in this laboratory (8). Cell culture media were from GIBCO. [^3H]P(Bu) $_2$ (specific activity, 6.4 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) and unlabeled P(Bu) $_2$ were from Lifesystems Company (Newton, MA). Other phorbol compounds were from Consolidated Midland (Brewster, NY). Teleocidin B was a gift of T. Sugimura. Epidermal growth factor (EGF) and fibroblast growth factor were from Collaborative Research (Waltham, MA). Platelet-derived growth factor, platelet-poor plasma derived serum, and lipoprotein-depleted human serum were gifts from Larry Witte and DeWitt Goodman. Human serum albumin was a gift from Van der Sande (New York Blood Center). Animal sera were obtained from normal animals. Pooled human serum was obtained from normal donors. Human amniotic fluid, human pregnancy urine, and human cerebrospinal fluid were identified by number code to preserve confidentiality. Rat embryo and adult rat liver extracts were prepared at 4°C by homogenizing the tissue in an equal volume of 0.3 M sucrose/10 mM Tris·HCl, pH 7.4, in a Polytron homogenizer. The homogenate was centrifuged at $10,000 \times g$ for 30 min and the supernatant fraction was centrifuged at $100,000 \times g$ for 60 min. The second supernatant fraction was then assayed for protein and for inhibition of [^3H]P(Bu) $_2$ binding.

P(Bu) $_2$ Binding Assay. CREF cells were grown in 5-cm plastic tissue culture dishes containing 5 ml of Dulbecco's modified Eagle's minimal essential medium plus 10% calf serum. Binding assays were conducted on 1 to 2×10^6 cells per dish. The monolayer was washed once with 5 ml of phosphate-buffered saline and 2 ml of assay buffer (2 vol of Dulbecco's medium plus 1 vol of buffered saline plus bovine serum albumin at 1 mg/ml) was added. For most experiments, the cell monolayer was then incubated for 60 min at 37°C prior to addition of 3 nM [^3H]P(Bu) $_2$ and possible inhibitors. After this addition, the cells were usually incubated for 30 min at 37°C . The monolayer was then rapidly washed three times, with a total of 15 ml of ice-cold assay

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Abbreviations: TPA, 12-O-tetradecanoylphorbol 13-acetate; P(Bu) $_2$, phorbol 12,13-dibutyrate; EGF, epidermal growth factor; ID $_{50}$, concentration required for 50% inhibition of binding.

buffer. The cells were solubilized for 2 hr at 37°C in 1 ml of 0.8% Triton X-100/0.02% EDTA/0.25% trypsin in buffered saline. The plates were then washed twice with 0.5 ml of 1% NaDodSO₄. The solubilizing solution and washings were combined and assayed for radioactivity in 15 ml of Hydrofluor scintillation fluid (National Diagnostics). [³H]P(Bu)₂ was supplied at approximately 8 μM in toluene. Aliquots were dried under a stream of nitrogen, redissolved in a small volume of dimethyl sulfoxide, and diluted to a convenient volume in assay buffer. Stock solutions of phorbol esters and related compounds were 2–20 mM in dimethyl sulfoxide. Serum fractions were diluted with buffered saline. Nonspecific binding was that observed in the presence of 50 μM unlabeled P(Bu)₂. In standard assays the nonspecific binding was approximately 30% of the total binding. All of the values presented are the means of duplicate determinations, which agreed within <10%. All experiments were repeated at least twice and gave similar results.

Fractionation of Serum. Human serum was applied to a 10 × 2.5 cm column of Blue Sepharose C1-6B equilibrated with phosphate-buffered saline and eluted with the same saline at 1 ml/min. The material bound to the column, which was mainly albumin (9), was then eluted with 0.5 M NaCl/50 mM Tris-HCl, pH 8.0. Protein content was monitored by absorbance at 280 nm and fractions were tested directly for inhibitory activity in a [³H]P(Bu)₂ binding assay. The major inhibitory activity eluted early from the column (prior to albumin) and was designated fraction 1. Fraction 1 was mixed with an equal volume of packed CM-Sephadex in 0.01 M sodium acetate buffer (pH 5.5). After 15 min at 0°C, the mixture was centrifuged and the supernatant fraction was reextracted with a second batch of CM-Sephadex. Material binding to the CM-Sephadex was eluted with 1.5 M NaCl/50 mM Tris-HCl, pH 7.4. Both fractions were dialyzed against phosphate-buffered saline and then assayed for inhibition of [³H]P(Bu)₂ binding. To estimate molecular weight, a 3-ml sample of fraction 1 was applied to a Sephadex G-100 column (26 × 1.5 cm) and eluted with buffered saline, and fractions were tested for inhibition of [³H]P(Bu)₂ binding. The column was calibrated with proteins of known molecular weights.

Protein concentrations were determined by the Lowry method (10). Serum fractions were also analyzed by NaDodSO₄/polyacrylamide gel electrophoresis on 10% acrylamide separation gel with 5% stacking gel (11).

RESULTS

When CREF cell monolayers were incubated with 3 nM [³H]P(Bu)₂ at 37°C specific binding occurred rapidly and reached a maximum within 10 min (Fig. 1A). Specific binding decreased slightly (24%) by 20 min but remained constant thereafter for at least 3 hr at 37°C. The decrease between 10 and 20 min was observed repeatedly but its significance is not known. At 4°C, specific [³H]P(Bu)₂ binding was considerably slower, requiring 3 hr to reach a maximum. The plateau value at 4°C was similar to that obtained at 37°C. In this study and in all of the subsequent studies, we measured total binding and nonspecific binding of [³H]P(Bu)₂ in parallel; nonspecific binding is that obtained in the presence of a vast excess (50 μM) of unlabeled P(Bu)₂. The latter value was subtracted from the total binding to obtain the specific binding. Nonspecific [³H]P(Bu)₂ binding was complete within 30 min, at either 37 or 4°C. At equilibrium binding of 3 nM [³H]P(Bu)₂, it accounted for less than one-third of the total cell-associated radioactivity.

When binding of [³H]P(Bu)₂ at 37°C was allowed to reach a plateau and the cell monolayer then was transferred to buffer lacking [³H]P(Bu)₂, the bound radioactivity was rapidly released from the cells. At 37°C most of the specifically bound [³H](Bu)₂ was released within 10 min. At 4°C, 70% of the specifically

FIG. 1. (A) Time course of specific binding of [³H]P(Bu)₂ to CREF cells. [³H]P(Bu)₂ binding was conducted at 37°C (▲) or 4°C (●). All values have been corrected for nonspecific binding which was determined in parallel assays. (B) Dissociation of [³H]P(Bu)₂ from CREF cells. [³H]P(Bu)₂ was allowed to bind to monolayers of CREF cells (2 × 10⁶ cells per dish) at 37°C for 30 min as in A. The assay buffer was then rapidly replaced with fresh assay buffer lacking [³H]P(Bu)₂ and the monolayers were incubated for varying time intervals at 37°C (▲) or 4°C (●). All values have been corrected for nonspecific binding.

bound [³H]P(Bu)₂ was released within 30 min and a slow release of the remaining radioactivity occurred thereafter (Fig. 1B).

Various compounds were tested at 37°C for possible inhibition of specific binding of [³H]P(Bu)₂ to CREF cells (Table 1). Phorbol and 4- α -phorbol didecanoate, which lack tumor-promoting activity in mouse skin and also lack activity in various cell culture assays (1–3), were unable to inhibit binding of [³H]P(Bu)₂ even at 1000 ng/ml. Conversely, the potent tumor-promoting phorbol ester TPA was a potent inhibitor of [³H]P(Bu)₂ binding and had an ID₅₀ (concentration required to inhibit specific [³H]P(Bu)₂ binding by 50%) of 4 ng/ml. Teleocidin B exerts a number of effects in cell culture similar to those of TPA (12) and is a potent tumor promoter on mouse skin (H. Fujiki, personal communication) but is not structurally related to the phorbol esters. We found it to be a potent inhibitor of [³H]P(Bu)₂ binding, with an ID₅₀ of 3 ng/ml. Mezerein is a macrocyclic plant diterpene that is structurally related to the phorbol esters and has many of the same effects as TPA in cell culture (2–4) but is considerably weaker than TPA when tested alone as a tumor promoter on mouse skin (3, 4). However, it is quite potent when used during the second phase of tumor promotion on mouse skin (14). We found it to be potent as an inhibitor of [³H]P(Bu)₂ binding to CREF cells (ID₅₀ = 20 ng/ml).

In monolayer cultures the binding of ¹²⁵I-labeled EGF is inhibited by TPA and P(Bu)₂ (4, 15–18). However, EGF was unable to inhibit the binding of [³H]P(Bu)₂ under similar conditions (Table 1). The amount of EGF used was more than 5 times the amount required to saturate EGF binding to its own receptor in this cell line (unpublished data). These results are

Table 1. Inhibition of [^3H]P(Bu) $_2$ binding

Compound	ID $_{50}$, ng/ml*
Phorbol	>1000
4- α -Phorbol didecanoate	>1000
TPA	4
Teleocidin B	3
Mezerein	20
EGF	>50
Fibroblast growth factor	>100
Platelet-derived growth factor	>100
Arginine vasopressin	>150
Lysine vasopressin	>300
Luteinizing-hormone releasing hormone	>500
Diazepam	>3 $\times 10^4$
Concanavalin A	>100
Wheat germ agglutinin	>100
4- α -Methyl glucoside	>3 $\times 10^7$

Specific binding of [^3H]P(Bu) $_2$ to CREF cells was assayed at 37°C for 30 min in the presence or absence of various concentrations of the indicated compounds added at time zero.

*ID $_{50}$, concentration of compound required to produce 50% inhibition of [^3H]P(Bu) $_2$ binding.

consistent with previous evidence that the phorbol ester inhibition of EGF binding is exerted via an indirect mechanism rather than by direct competition for binding to EGF receptors (15–18). Two other growth factors—platelet-derived growth factor and fibroblast growth factor, had no effect on [^3H]P(Bu) $_2$ binding. Arginine and lysine vasopressins, luteinizing-hormone releasing hormone, diazepam, concanavalin A, wheat germ agglutinin, and 4- α -methyl glucoside also were negative (Table 1).

The binding of [^3H]P(Bu) $_2$ to CREF cell monolayers was characterized by Scatchard analysis (19). Nonspecific binding was linear with [^3H]P(Bu) $_2$ concentration. To conserve [^3H]P(Bu) $_2$ and to maintain a low background of nonspecific binding, most of our binding assays were done with a constant concentration (2–4 nM) of [^3H]P(Bu) $_2$ and increasing amounts of unlabeled P(Bu) $_2$. With this method, we found that Scatchard analysis of the binding was nonlinear (Fig. 2). This was consistent with multiple binding sites or cooperativity among binding sites. If the first interpretation is assumed to be correct, there appear to be at least two classes of binding sites. Our data indicate that the high-affinity sites have a K_d of about 8 nM, and that there are 1.6×10^5 of these receptors per cell. In assays in which a preincubation was not carried out, the number of high-affinity sites was about 1×10^5 per cell. The low-affinity sites have a K_d of 710 nM and there are about 3×10^6 of these sites per cell. The large number of the latter type of sites suggests that they are not specific phorbol ester receptors but are structural elements in the cell to which these compounds also happen to bind.

We found that there was at most a 3-fold variation in the number of high- or low-affinity sites per cell when cultures were examined at various stages—i.e., exponential growth, subconfluence, or confluence. Most of the present studies were performed with subconfluent cultures.

Inhibition of [^3H]P(Bu) $_2$ Binding by Serum. Preincubation of cultures in the absence of serum at 37°C for 60 min prior to the addition of [^3H]P(Bu) $_2$ led to a 2-fold increase in number of available [^3H]P(Bu) $_2$ receptors. This suggested that the serum in which the cells had been grown contained an inhibitory factor. We found that addition of either calf or fetal calf serum to the assay system caused a concentration-dependent inhibition

FIG. 2. Scatchard plot of specific binding of [^3H]P(Bu) $_2$ to CREF cells. The results from two separate experiments are shown (● and ○). Binding assays were performed on approximately 2×10^6 cells per dish at 37°C for 30 min. Bound [^3H]P(Bu) $_2$ (B) is expressed as pmol per 10^6 cells and is corrected for nonspecific binding. The concentration of [^3H]P(Bu) $_2$ in the binding solution (F) is in $\mu\text{mol/liter}$. The data are consistent with two binding sites: ---, $K_{d1} = 7.6$ nM, at 1.6×10^5 sites per cell; and - · - · -, $K_{d2} = 710$ nM, at 2.8×10^6 sites per cell.

of [^3H]P(Bu) $_2$ binding to CREF cells, with 50% inhibition occurring at a protein concentration of about 11 mg/ml (Fig. 3; Table 2). Pooled normal human serum had an even greater effect, producing 50% inhibition of [^3H]P(Bu) $_2$ binding at a concentration of about 4 mg/ml (Fig. 3). Human serum from platelet-depleted plasma was also active, suggesting that the inhibitory activity is not derived from platelets. This is consistent with the lack of activity of platelet-derived growth factor (Table 1). Lipoprotein-depleted human serum was also active, suggesting that the effect did not reflect nonspecific trapping of the [^3H]P(Bu) $_2$ in serum lipids. Other biologic fluids that contained significant activity included rhesus monkey serum, pregnant rat serum, human amniotic fluid, rat embryo and rat liver homogenates (Table 2). Cerebrospinal fluid and concentrated urine were negative. Although purified serum albumin did inhibit [^3H]P(Bu) $_2$ binding, this occurred only with concentrations 10-fold greater than those required with an equivalent amount of serum.

When CREF cells were incubated in serum the inhibition of [^3H]P(Bu) $_2$ binding was immediate and sustained. When cells

FIG. 3. Inhibition of specific binding of [^3H]P(Bu) $_2$ to CREF cells by various physiological fluids. ●, Pooled human serum (two different batches); ○, calf serum; ▲, human amniotic fluid; ■, rat serum. All data are corrected for nonspecific [^3H]P(Bu) $_2$ binding. The test materials were added to the assay system at time zero, simultaneously with the addition of [^3H]P(Bu) $_2$. Assays were incubated at 37°C for 30 min.

Table 2. Inhibition of [^3H]P(Bu) $_2$ binding to CREF cells by various physiologic fluids

Test substance	Approximate ID $_{50}$, mg/ml*
Calf serum	11
Fetal calf serum	11
Pooled human serum	4
Pregnant human serum	2
Platelet-depleted human serum	4
Lipoprotein-depleted human serum	4
Rhesus monkey serum	8
Rat serum	1
Human amniotic fluid	2
Rat embryo homogenate	0.3
Rat liver homogenate	1
Human cerebrospinal fluid	NA
Rhesus monkey cerebrospinal fluid	NA
Human pregnancy urine	NA
Human serum albumin	25

* NA, no activity detected when tested at a final concentration of 50% test substance in the binding assay buffer. Cerebrospinal fluid was used as such. Urine was first dialyzed and then concentrated 10-fold by evaporation.

were preincubated in the presence of 50% serum for 60 min and binding of [^3H]P(Bu) $_2$ was then measured after the medium was replaced with serum-free medium, a gradual recovery of [^3H]P(Bu) $_2$ binding was seen which reached a maximum after about 2 hr (data not shown here). Thus, inhibition of [^3H]P(Bu) $_2$ binding by serum is reversible. The effect of human serum and amniotic fluid on [^3H]P(Bu) $_2$ binding does not require cellular metabolic functions or intact cells because we found that both serum and amniotic fluid inhibited [^3H]P(Bu) $_2$ binding in a crude membrane preparation to a similar extent as with intact cells. In addition, human serum was able to inhibit [^3H]P(Bu) $_2$ binding at both 4 and 37°C. The latter findings are in contrast to the inhibition of EGF-receptor binding by tumor-promoting phorbol esters, which appears to be due to an indirect effect rather than direct competition for EGF receptors (15–18).

Fractionation of Serum. Human serum was fractionated by affinity chromatography, ion exchange, and gel filtration to partially purify the above factor (Table 3). In the chromatography on a Blue Sepharose CL-6B column, which retains albumin (9), most of the inhibitory activity was found in the void volume. Analysis of this material by NaDodSO $_4$ /polyacrylamide gel electrophoresis indicated that it was virtually free of albumin. Fractionation on the Blue Sepharose column led to a 3- to 8-fold purification.

Further purification of the inhibitory factor was achieved by treating the Blue Sepharose fraction with CM-Sephadex at pH

5.5 in a batchwise centrifugation technique. The inhibitory activity was found in the supernatant fraction (i.e., it did not bind to the CM-Sephadex). It was enriched about 135-fold, with an ID $_{50}$ of 0.04 mg/ml compared to 5.4 mg/ml for the original unfractionated serum (Table 3). The total yield of inhibitory activity actually increased about 3-fold after these two partial purification steps, suggesting that whole serum contains a substance that prevents this factor from exerting its full activity. Although gel electrophoresis indicated that α_1 -antitrypsin was present in the CM-Sephadex fraction, a commercial preparation of α_1 -antitrypsin lacked inhibitory activity even when tested at a protein concentration 5 times the ID $_{50}$ of this fraction (Table 2). Fractionation on a Sephadex G-100 column indicated that the major peak of inhibitory activity had a molecular weight of about 60,000. A minor peak of inhibitory activity with a very low molecular weight was also detected but this material has not been studied further. The major factor present in serum remained active after precipitation with cold 80% ethanol but was inactivated by heating at 70°C for 10 min.

DISCUSSION

The present results provide direct evidence that intact rat embryo fibroblasts contain specific high-affinity receptors for [^3H]P(Bu) $_2$ and related phorbol ester tumor promoters. Our findings complement the studies by Blumberg and colleagues (6, 7) who recently demonstrated receptors for [^3H]P(Bu) $_2$ in crude membrane preparations of chicken embryo fibroblasts and mouse epidermis.

Our finding that, in intact cell monolayers [^3H]P(Bu) $_2$ binding at 37°C rapidly reaches a plateau and is also rapidly reversible provides evidence that these receptors are on the cell surface, presumably associated with the plasma membrane. Using our intact cell assay, we have also detected similar [^3H]P(Bu) $_2$ receptors in various cell types including Friend erythroleukemia cells, mouse embryo fibroblast cell line 10T $\frac{1}{2}$, rat liver epithelial cell line K22, and an adenovirus-transformed rat embryo cell line (unpublished data). The intact cell assay for phorbol ester receptors described in the present study should facilitate further studies on the physiology of this receptor–ligand system and its probable role in mediating the various biologic effects of this interesting class of compounds.

The specificity of [^3H]P(Bu) $_2$ binding was demonstrated by the inability of a non-tumor-promoting phorbol ester and the parent phorbol to compete for [^3H]P(Bu) $_2$ binding sites. On the other hand, the potent tumor promoters TPA and teleocidin B competed strongly with [^3H]P(Bu) $_2$ for receptor binding. Teleocidin B shares a number of membrane-related effects with TPA (20). Blumberg and colleagues (6, 7) have shown that inhibition of [^3H]P(Bu) $_2$ binding to crude membrane preparations by a series of plant diterpenes also generally correlates with their tumor-promoting activity on mouse skin.

A Scatchard analysis of [^3H]P(Bu) $_2$ binding to intact CREF cells gave a biphasic curve compatible with two binding sites, one with a K_d of 8 nM and the other with a K_d of 710 nM. It is also possible that the nonlinearity arises from negative cooperativity in binding, and further studies are required to exclude this possibility. The K_d value of 8 nM for the high affinity sites is in the range of that observed for other cell surface receptors (i.e., EGF) (4). We estimate that there are about $1\text{--}2 \times 10^5$ high-affinity [^3H]P(Bu) $_2$ sites per cell, which is about 10-fold higher than the number of EGF receptor sites that we find on CREF cells (unpublished data). Our values for the K_d and total number of high-affinity [^3H]P(Bu) $_2$ receptors in these intact rat fibroblasts are in good agreement with the values obtained with crude membrane preparations from avian fibroblasts or mouse epidermis (6, 7). Thus, various species and cell types contain

Table 3. Inhibition of [^3H]P(Bu) $_2$ binding by serum fractions

Step	ID $_{50}$, mg/ml protein	Purification, -fold
Pooled human serum	5.4	1
Dialyzed human serum	5.0	—
Blue Sepharose fraction 1	0.7–1.7	3–8
CM-Sephadex fraction 1*	0.04	135
Sephadex G-100 peak fraction*	0.75	7

* Starting material was Blue Sepharose fraction 1.

similar membrane-associated receptors for the phorbol esters, a finding consistent with evidence that diverse cell types show similar structure-function requirements when tested for biologic responses with a series of phorbol esters and related plant diterpenes (1-3).

Several known growth factors and certain other membrane active compounds failed to inhibit [^3H]P(Bu) $_2$ -receptor binding. However, calf, human, rhesus monkey, and rat sera were highly inhibitory, as were certain other biologic fluids and tissue extracts (Table 2). We have obtained evidence that serum contains a specific factor(s) that inhibits [^3H]P(Bu) $_2$, that the major factor is a neutral or slightly acidic protein with a molecular weight in the range of 60,000, and that this factor is not albumin. This factor has been purified about 135-fold. Once a more purified material has been obtained, it should be possible to determine if this substance exerts its inhibitory effect by competing directly with [^3H]P(Bu) $_2$ for binding to the same set of cellular receptors, by binding [^3H]P(Bu) $_2$ itself, or by some other mechanism. It will also be of interest to determine whether this substance induces biologic effects similar to those of the phorbol esters or whether it antagonizes the action of these tumor promoters.

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