Modulation of Protein Kinase C Activity and \([^{3}H]\)Phorbol 12,13-Dibutyrate Binding by Various Tumor Promoters in Mouse Brain Cytosol

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ABSTRACT

Using protein kinase C partially purified from mouse brain cytosol, we examined the effect of a number of phorbol ester and nonphorbol tumor promoters on protein kinase C enzymatic activity and \([^{3}H]\)phorbol 12,13-dibutyrate binding. Mezerein and phorbol 12-retinoate 13-acetate, second stage tumor promoters, as well as the weak tumor promoter 4-O-methylphorbol 12-myristate 13-acetate stimulated kinase activity to the same extent as did the complete tumor promoter phorbol 12-myristate 13-acetate. In contrast, the nonphorbol ester tumor promoters anthralin, cantharidin, benzoyl peroxide, and 7-bromomethylbenz(a)anthracene did not affect kinase activity. The unsaturated fatty acids palmitoleic, oleic, linoleic, linolenic, and arachidonic acids, some of which have been reported to be weak tumor promoters, stimulated protein kinase C activity in the presence of phospholipids, as well as causing some activation in the absence of phospholipids. The saturated fatty acids butyric, lauric, myristic, and palmitic acids had relatively little effect. The fatty acids showed generally similar structure-activity relationships for inhibition of \([^{3}H]\)phorbol 12,13-dibutyrate binding as for stimulation of kinase activity. The unsaturated fatty acids typically decreased binding levels for the reconstituted aporeceptor, while the saturated fatty acids did not. The nature of this inhibition was explored in the case of arachidonic acid. Scatchard analysis demonstrated decreases in both the maximum number of binding sites as well as the apparent binding affinity, indicative of a complex mechanism. As expected for a lipophilic ligand, the effect of the arachidonic acid was reduced in the presence of a complex mechanism. As expected for a lipophilic ligand, the effect of the arachidonic acid was reduced in the presence of

INTRODUCTION

The phorbol esters are the most potent class of tumor promoters for mouse skin (11, 12). In addition, these compounds induce a wide variety of responses in many biological systems. This laboratory and others (7, 9, 15, 37, 42, 44) have used \([^{3}H]\)PDBu to demonstrate specific high affinity receptors for phorbol esters in many different intact cells and tissue preparations. The structure-activity requirements for binding are consistent with the receptors mediating a number of the biological responses to the phorbol esters, including their tumor-promoting activity.

Subcellular localization studies have demonstrated \([^{3}H]\)PDBu binding activity in both the particulate and cytoplasmic fractions (2, 27, 38). The distribution is dependent upon the conditions of cell lysis; lysis in the presence of chelators increases the \([^{3}H]\)PDBu binding activity recovered in the 100,000 × g supernatant fraction (17, 33). The cytoplasmic receptor requires the addition of phospholipids to reconstitute binding activity, and this receptor copurifies with PKC (2, 27, 38).

PKC requires calcium and phospholipids for enzymatic activity (see Ref. 45 for review). In vitro, diacylglycerols stimulate PKC activity by shifting the calcium concentration dependency curve to lower calcium concentrations. Castagna et al. (3) demonstrated that PMA stimulates PKC activity in a manner similar to diacylglycerols, and Sharkey et al. (41) have shown that diacylglycerols competitively inhibit phorbol ester binding. Further, the structure activity requirements of phorbol diesters for biological activity in mouse skin are similar to those for PKC activation (3).

In addition to the phorbol esters, a number of non-phorbol ester compounds including anthralin, benzoyl peroxide, and fatty acids act as complete tumor promoters in mouse skin (see Ref. 5 for review). These structurally unrelated tumor promoters, however, are typically 10,000 to 500,000 times less potent than PMA (6). Previous studies (6) have demonstrated that the non-phorbol tumor promoters do not in general induce similar biological effects to the phorbol esters in chick embryo fibroblasts and do not inhibit \([^{3}H]\)PDBu binding.

In the present study, we have examined the effect of a number of tumor-promoting agents on PKC activity. The mode of action of the various non-phorbol ester tumor promoters is unknown, and modulation of PKC activity represents one mechanism by which some of these compounds may promote tumor growth. For compounds which this laboratory had shown previously not to inhibit phorbol ester binding to the membrane receptor, we wished to determine whether they might activate PKC directly through interaction at a different site. In addition, in the case of the highly lipophilic fatty acids, their activity to inhibit PDBu binding had not been examined previously because of potential problems of enhanced nonspecific binding. Using the partially purified reconstituted aporeceptor, this technical problem can now be minimized.

A third issue investigated by these experiments was the concentration dependency relationship of PKC activation by PMA. This topic was of interest because of several literature reports demonstrating bell-shaped dose-response curves for biological effects of PMA. These observations include the induction of sister chromatid exchange in mouse 10T1/2 cells (32), the enhancement of colony formation in transformed human leukocytes (49), and the reduction in fibroblast cloning efficiency (25). We wished to determine whether a biphasic concentration de-
DEPENDENCY CURVE OF PMA ACTIVATION OF PKC COULD ACCOUNT FOR THESE OBSERVATIONS.

MATERIALS AND METHODS

Sources of materials were as follows: [3H]PDBu (specific activity, 12.2 to 13.4 Ci/mmol) and [γ-32P]ATP (specific activity, 1000 Ci/mmol), New England Nuclear (Boston, MA); nonradioactive phorbol esters, Chemicals for Cancer Research (Eden Prairie, MN); PC, Supelco (Bellevonte, PA); DEAE-cellulose (DE52) and phosphocellulose paper, Whatman (Clifton, NJ); anthralin, Ruger Chemical Co. (Irvington, NJ); benzoyl peroxide, Chemical Dynamics Corp. (South Plainfield, NJ); and PRA, LC Services Corp. (Woburn, MA). DPB was prepared by Dr. Joseph Dunn (8). 7-Bromomethylbenz(a)anthracene was a gift from Dr. Anthony Dipple, Frederick Cancer Research Facility, Frederick, MD. All other materials were from Sigma Chemical Co. (St. Louis, MO).

Tissue Preparation. Whole brains from 6- to 8-week-old female CD-1 mice (Charles River Breeding Laboratories) were homogenized with a Potter-Elvehem homogenizer at 4 °C in 1 volume of 20 mM Tris-Cl (pH 7.4) which contained 2 mM EDTA, 10 mM EGTA, and 0.25 M sucrose. The homogenate was centrifuged at 100,000 × g for 60 min at 4 °C.

Column Chromatography. The 100,000 × g supernatant was applied to a DE52 column (2.5 x 15 cm) equilibrated with 20 mM Tris-Cl (pH 7.4) containing 2 mM EDTA, 5 mM EGTA, and 5 mM dithiothreitol (Buffer B), and the column was washed with 450 ml Buffer B. The enzyme was eluted from the column with a 300-ml linear concentration gradient of NaCl (0 to 0.3 M) in Buffer B, and fractions of 10 ml were collected. Each fraction was assayed for protein kinase C activity in the presence of 1 mM CaCl₂ and PS (160 µg/ml). Peak fractions were pooled and stored at −20 °C in the presence of 50% glycerol.

For subsequent assays, an aliquot of the DE52-purified preparation was desalted by chromatography on 2 PD-10 columns immediately before use in order to remove chelators. The enzyme preparation (2.5 ml) was applied to a PD-10 column equilibrated with 20 mM Tris-Cl (pH 7.4) at 4 °C. The column was washed with 0.5 ml Tris-Cl, and the enzyme was eluted with 2.5 ml Tris-Cl. The 2.5-ml-column eluate was applied to a second PD-10 column equilibrated with Tris-Cl and eluted with 3.0 ml Tris-Cl.

Binding Assay. [3H]PDBu binding was determined in a 250-µl incubation mixture containing [3H]PDBu (3 to 100 nm), 50 mM Tris-Cl (pH 7.4), 1 mM CaCl₂, 7.5 mM magnesium acetate, bovine γ-globulin at 4 mg/ml, PS:PC (1:2) at 60 or 600 µg/mg, 2 to 10 µg DE52-purified enzyme, and other ligands as specified. Fatty acids were dissolved in methanol and diluted with 50 mM Tris-Cl (pH 7.4) prior to addition to the assay. Dilutions of [3H]PDBu and PDBu were made in 20 mM Tris-Cl (pH 7.4) containing BSA (1 mg/ml). Anthralin and cantharidin were dissolved in dimethyl sulfoxide and diluted with 20 mM Tris-Cl (pH 7.4) prior to addition to the assay. Benzoyl peroxide (dissolved in the acetone, then diluted in 20 mM Tris-Cl) was preincubated with the phospholipid mixture for 30 min at 37 °C before addition to the kinase assay. 7-Bromomethylbenz(a)anthracene was dissolved in dry benzene and mixed with aliquots of PS and PC (in chloroform). The solvent was evaporated under nitrogen gas, and the mixture was sonicated as described above. Incubations were carried out in 1.5-ml tubes for 5 min at 30 °C. Immediately after incubation, the tubes were placed on ice, aliquots (25 µl) were spotted onto 2- x 2-cm squares of phosphocellulose paper, and the paper was washed 3 times in water, rinsed in acetone and then in petroleum ether, and dried (48). Radioactivity was assayed in 4 ml of Aquasol (New England Nuclear). Enzymatic activity is expressed as pmol 32P incorporated per 5 min.

RESULTS

Mouse brain cytosol partially purified by DE52 chromatography was used to assay PKC activity. PMA increased PKC activity with maximal stimulation at approximately 100 nM PMA (Chart 1). Although PMA stimulation of PKC activity has been reported previously by several laboratories, to our knowledge, no group has examined the effect of PMA at concentrations greater than 200 nM. Biphasic curves for biological effects or PMA have been demonstrated in several literature reports (25, 32, 49). In order to determine whether these effects could be explained by a biphasic concentration dependency curve of PMA activation of PKC, we examined much higher concentrations of PMA than have been reported previously. We found that PKC activity did not change further with increasing concentrations of up to 10 µM PMA.

The weakly promoting phorbol ester, 4-O-methyl-PMA, stimulated PKC activity as did the second-stage promoters, mezerein and PRA (Table 1). DPB is an inflammatory but relatively nonpromoting phorbol ester that binds to a subclass of the [3H]-PDBu binding sites in mouse skin membrane preparations (8).
Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>PKC activity (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMA (100 nM)</td>
<td>100 ± 3a</td>
</tr>
<tr>
<td>Mezerein (3000 nM)</td>
<td>102 ± 1</td>
</tr>
<tr>
<td>PRA (3000 nM)</td>
<td>95 ± 6</td>
</tr>
<tr>
<td>DPB (3000 nM)</td>
<td>96 ± 2</td>
</tr>
<tr>
<td>4-O-Methyl-PMA (3000 nM)</td>
<td>116 ± 4</td>
</tr>
<tr>
<td>Anthralin (10 µM)</td>
<td>1 ± 5</td>
</tr>
<tr>
<td>Cantharidin (10 µM)</td>
<td>12 ± 5</td>
</tr>
<tr>
<td>Benzoyl peroxide (100 µg/ml)</td>
<td>0.2 ± 9</td>
</tr>
<tr>
<td>7-Bromomethylbenz[a]anthracene (100 µg/ml)</td>
<td>0.2 ± 9</td>
</tr>
<tr>
<td>Oleic acid (350 µM)</td>
<td>230 ± 6</td>
</tr>
</tbody>
</table>

* Average ± range of duplicate determinations within a single experiment. Similar results were obtained in 2 additional experiments.

This phorbol ester also increased PKC levels. All 4 compounds increased enzyme levels to the same maximal degree as did 200 nM PDBu, although concentrations of up to 3000 nM were required for maximal stimulation.

A number of non-phorbol ester tumor promoters were tested for their ability to stimulate PKC activity in the presence of phospholipids. Anthralin, cantharidin, benzoyl peroxide, and 7-bromomethylbenz[a]anthracene all were unable to stimulate PKC activity (Table 1). Fatty acids, such as oleic acid, have weak tumor-promoting activity. When added to enzyme preparations at 350 µM, oleic acid markedly stimulated PKC activity. The level of stimulation by oleic acid was over 2-fold that caused by 200 nM PDBu.

Various fatty acids differed in their ability to stimulate PKC activity. The concentration dependency curves for representative fatty acids, palmitic, palmitoleic, and arachidonic acids, are shown in Chart 2A. The activation concentration dependency curves for the other saturated and unsaturated fatty acids examined (see Table 2) corresponded closely to those of palmitic and palmitoleic acids, respectively, and thus have not been included in Chart 2. The saturated fatty acids, such as palmitic acid, had relatively little effect on PKC activity, either in the presence or absence of phospholipids.

Table 2 is a summary of the effects of a number of fatty acids on both PKC and [³H]PDBu binding activity. Since maximal effects on PKC activity occurred at approximately 300 µM (100 µg/ml) for the fatty acids, as shown in Chart 2A, the effect of this concentration was used in compiling Table 2. In the absence of phospholipids, the saturated fatty acid butyric, like palmitic acid, was ineffective at stimulating PKC activity (Table 2). Both lauric and myristic acids increased PKC activity somewhat in the presence of phospholipids, which were saturated fatty acids, oleic, linoleic, and arachidonic acids all increased PKC levels more than did the saturated fatty acids, in both the presence and absence of PS:PC mixture. Fatty acids increased PKC activity in the presence of 200 nM PDBu as well; the unsaturated fatty acids and lauric acid increased PKC activity to over twice the level as did 200 nM PDBu alone, while myristic and palmitic acids increased PKC activity between 40 and 70%.

Since the unsaturated fatty acids markedly stimulated PKC activity, both in the presence of phospholipids alone and with phospholipids plus PDBu, we asked whether these compounds might affect [³H]PDBu binding activity as well. The fatty acids showed generally similar but not identical structure-activity relationships for inhibiting [³H]PDBu binding in the presence of...
PS:PC as for stimulating kinase activity. Saturated fatty acids, such as palmitic acid, had no effect on [\textsuperscript{3}H]PDBu binding activity (Chart 2B). Unsaturated fatty acids, however, inhibited binding levels in a concentration-dependent manner. The \( K_i \) for inhibition by palmitoleic, linoleic, and arachidonic acids, determined from the 50% inhibition dose, was approximately 50 \( \mu M \).

The summary of the fatty acid effects on binding activity is shown in Table 2. The percentage of inhibition of binding by the unsaturated fatty acids ranged from a low of 10% with oleic acid to over 90% with linolenic acid. In the absence of phospholipids, [\textsuperscript{3}H]PDBu binding activity was less than 2 pmol/mg, but the addition of several of the fatty acids, both saturated and unsaturated, significantly increased binding levels.

In order to explore the mechanism of binding inhibition by the fatty acids, arachidonic acid was chosen as a representative unsaturated fatty acid, and Scatchard analysis was carried out (Chart 3). Arachidonic acid decreased both the affinity of [\textsuperscript{3}H]PDBu as well as the maximal extent of binding at saturation. Total binding sites decreased from the control value of 221 ± 6 (SE) pmol/mg (n = 6) to 154 ± 1 pmol/mg (n = 3) in the presence of 260 \( \mu M \) arachidonic acid (80 \( \mu g/ml \)) while the apparent \( K_a \) decreased approximately 10-fold, from 0.58 ± 0.07 nm to 5.22 ± 0.33 nm.

We reasoned that the highly lipophilic fatty acids will interact with phospholipids, and thus, their effects may depend on not only absolute concentrations but also their proportion relative to the phospholipids. Therefore, we also carried out Scatchard analysis in the presence of a higher concentration of PS:PC (Chart 4). As predicted, we found that increasing the PS:PC concentration 10-fold in the presence of 260 \( \mu M \) arachidonic acid (80 \( \mu g/ml \)) decreased the extent of inhibition. The total binding sites increased to 194 ± 8 pmol/mg (n = 2), which was similar to control levels (210 ± 18, n = 2), and the binding affinity increased somewhat as well, to 1.75 ± 0.6 nm (n = 2), although it still did not reach the control value (0.64 ± 0.06 nm, n = 2).

**DISCUSSION**

In these studies, we used the phospholipid mixture PS:PC (1:2) to reconstitute enzymatic activity. We chose these phospholipids, rather than PS alone, in order to mimic more closely the in vivo phospholipid environment (13, 47). This mixture results in lower basal PKC activity, compared to PS alone (19), thus enhancing the degree of stimulation by PDBu and 1,2-diolein. Reconstitution with the PS:PC mixture in place of PS had little effect on the binding affinity of [\textsuperscript{3}H]PDBu for the cytosolic receptor (data not shown).

Using the PS:PC system, we demonstrated that the concentration dependency curve for PMA stimulation of PKC activity increased with increasing PMA concentrations, attaining a plateau level by 100 \( \mu M \) PMA. Further increases in PMA concentrations of up to 10 \( \mu M \) had no further effects. These findings...
suggest that the biphasic curves are not a result of inhibition of PKC at high PMA concentrations. Instead, the biphasic response curves may be the result of more than one receptor mediating the effects of PMA or one receptor in various affinity states or with different degrees of accessibility; both heterogeneity in phorbol ester binding (4, 8, 15) and modulation of binding affinity and PKC activity by phospholipids (19, 24) have been demonstrated.

A number of nonphorbol esters have tumor-promoting activity (40). The mode of action of these tumor promoters is unknown, and thus, it was of interest to examine the effect of several of these compounds on PKC activity. Neither anthralin nor cantharidin inhibited [3H]PDBu binding in chick fibroblasts (7), and neither compound affected PKC activity in our in vitro assays. 7-Bromo-12-methylbenz(a)anthracene is an effective, complete carcinogen in mouse skin, and it acts as a tumor promoter after initiation with 7,12-dimethylbenz(a)anthracene as well (39). The promoter benzoyl peroxide, a free radical-generating compound, induces several similar biological effects as the phorbol esters, such as inhibition of metabolic cooperation (43). None of these nonphorbol promoters activated PKC in the presence of phospholipids. In addition, the compounds did not affect PDBu stimulation of the enzyme (data not shown). On the basis of their inability to inactivate PKC, our results indicate that these promoters need not act directly on the PDBu receptor:PKC. These agents may affect PKC activity at another step in the pathway, such as modulating phospholipase C activity and diacylglycerol formation (18); alternatively, the may act through an entirely different pathway.

Fatty acids represent another class of compounds with tumor-promoting activity. Undiluted oleic acid as well as oleic and lauric acids dissolved in chloroform exhibited significant tumor-promoting properties when applied to mouse skin 6 times/week. The saturated fatty acids, stearic and palmitic, did not induce tumor growth (14). In addition to their tumor-promoting activity, several of the fatty acids have been shown to induce other similar biological effects as the phorbol esters. For example, lauric acid stimulated deoxyglucose transport in chick embryo fibroblasts (6), and butyric acid induced the expression of Epstein-Barr virus early antigens in human lymphoblasts (21). In our experiments, lauric acid affected PKC and [3H]PDBu binding activity more than the other saturated fatty acids, consistent with its reported biological effects. Oleic acid was consistently poor at inhibiting [3H]PDBu binding activity, although it stimulated PKC activity. Conversely, linoleic acid was a good inhibitor of binding activity but a weak stimulator of PKC. The reason for these differences is not clear, although these results were duplicated with several different preparations of both oleic and linoleic acid.

We found that the unsaturated fatty acids both stimulated PKC activity and inhibited [3H]PDBu activity. Since our first report (26) of these findings and during the preparation of this paper, McPhail et al. (30) also have reported that fatty acids stimulate PKC activity in neutrophils. In contrast to our results, however, they found that high concentrations of fatty acids inhibit PKC activity. Differences in experimental systems may explain the concentration dependency curves; McPhail et al. used detergent extracts from neutrophils, while we used PKC partially purified from mouse brain, in the absence of any detergents. Although Kishimoto et al. (22) reported that fatty acids do not affect PKC activity, they may need to have examined low concentrations of the compounds. Our results demonstrate that concentrations greater than 30 μM are required to stimulate PKC activity.

In the presence of phospholipids, the unsaturated fatty acids increased PKC levels over 2-fold, compared to PDBu stimulation, and, except for oleic acid, inhibited [3H]PDBu binding by 50 to 90%, whereas the saturated fatty acids did not. Relatively little is known about the effects of free fatty acids upon membrane structure. Fluorescence polarization and conformational binding studies have shown that free fatty acids readily intercalate into lipid vesicles, resulting in perturbation of the phospholipid structure (1, 20, 23). cis-saturated fatty acids disorder the lipids to a much greater extent than do unsaturated fatty acids. As in our studies, a number of biological activities of fatty acids distinguish between saturated and unsaturated acids and may reflect the ability of the fatty acid to perturb lipid structure.

We found from Scatchard analysis that arachidonic acid affected both the binding affinity and total number of binding sites, consistent with the complex perturbation of the interactions between [3H]PDBu, aporeceptor, and phospholipid. Addition of higher concentrations of phospholipids reduced the inhibition, suggesting that the fatty acid:phospholipid ratio is an important factor in determining its activity. Previously, we had shown that the activity of diacylglycerols was directly related to their mole fraction in the phospholipids (41).

The fatty acid modulation of PKC and [3H]PDBu binding activity occurred in the absence of phospholipids as well as in the presence. Since both cytosolic activities are phospholipid dependent, these results may reflect the ability of the fatty acids to interact with hydrophobic sites on PKC, allowing activation of the kinase and binding of [3H]PDBu. We have argued previously that diacylglycerols are endogenous phorbol ester analogues (41). Although physical techniques may be required to determine whether the appropriate fatty acids interact at the phorbol ester binding site, our evidence strongly argues that this cannot be their only action. (a) Maximal [3H]PDBu binding as well as binding affinity is reduced. (b) The fatty acids give greater stimulation of PKC activity than do the phorbol ester, and the combination of the 2 ligands gives greater stimulation than either alone. (c) As described above, certain fatty acids in the absence of phospholipids can partially reconstitute the aporeceptor to permit binding. (d) Variation is found in the relative abilities of the fatty acids to reconstitute binding and to stimulate PKC. None of these results would have been expected for a true analogue.

A regulatory role of fatty acids in cellular metabolism has been documented in detail. The concentrations of fatty acids used here are in the range found to be active in these other studies. In vivo, fatty acids have been shown to inhibit glycerol 3-phosphate dehydrogenase in rat liver (29), adenylyl cyclase in rat adipose tissue (28), and glycerophosphate acyltransferase in yeast (31). Unsaturated fatty acids stimulate phosphatidylinositol phosphodiesterase in rat brain and liver (16, 46), CA2+-ATPase in erythrocyte membranes (34), and CTP:phosphocholine cytidylyltransferase in rat lung (10). In vitro, Pelech et al. (35) demonstrated that fatty acids promote translocation of CTP:phosphocholine cytidylyltransferase from the cytosol to the microsomal membrane, where it is then activated by the lipid environment.

Our results demonstrate that, in vitro, fatty acids modulate PKC and phorbol ester receptor binding activities; in vivo, these compounds may also play a role in cellular regulation of PKC.
REFERENCES


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