Correlation of the Antiproliferative Action of Diphenylmethane-Derivative Antiestrogen Binding Site Ligands with Antagonism of Histamine Binding but not of Protein Kinase C-mediated Phosphorylation

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ABSTRACT

The nonestrogen receptor-mediated antiproliferative action of antiestrogen binding site (AEBS) ligands, including triphenylethylene antiestrogens and phenothiazines, has been linked to their ability to inhibit protein kinase C (PKC). Recent studies indicate that some diphenylmethane derivatives inhibit growth, are potent AEBS ligands, and antagonize histamine binding at an AEBS-related histamine site different from H₁ and H₂. Three novel diphenylmethane derivatives, N,N-diethyl-2-[4-(phenylmethyl)phenoxy]ethanamine-HCl (DPPE), 4-decanoyl-DPPE (dec-DPPE), and 4-benzylphenyl decanoate (BPD) were studied in an attempt to determine whether PKC or histamine interactions best correlate with their antiproliferative effects. Platelet aggregation and the phosphorylation of a platelet M, 47,000 protein (p47) induced by phorbol 12-myristate-13-acetate (PMA) represent two processes mediated by PKC. DPPE inhibits PMA-induced aggregation [50% inhibitory concentration (IC₅₀) = 3.12 ± 2.4 (SEM) x 10⁻⁴ M] but does not significantly inhibit either PMA-induced phosphorylation of M, 47,000 protein (IC₅₀ > 500 x 10⁻⁴ M) or, binding of [³H]phorbol dibutyrate to platelets. dec-DPPE is a more potent inhibitor of PMA-induced platelet aggregation (IC₅₀ = 18.8 ± 0.7 x 10⁻⁴ M), a weak inhibitor of M, 47,000 phosphorylation (IC₅₀ = 80 - 200 x 10⁻⁴ M), but is without effect on [³H]phorbol dibutyrate binding. BPD, which lacks the alkylaminoethoxy side chain necessary for binding to the AEBS/DPPE site, is devoid of anti-PKA effects. These results are compared to the inhibition of [³H]histamine binding in rat cortex membranes (Kᵢ = 0.0 ± 0.0 M) for DPPE = 0.63 ± 0.62 x 10⁻⁴ M; Kᵢ for dec-DPPE = 6.6 ± 3.5 x 10⁻⁴ M; BPD is inactive) and growth inhibition of MCF-7 cells (IC₅₀ value for DPPE = 4.5 x 10⁻⁴ M; IC₅₀ value for dec-DPPE = 1.5 x 10⁻³ M; BPD is ineffective at all concentrations tested). Thus, while dec-DPPE is a more potent inhibitor of PKC-mediated phosphorylation, DPPE is a more potent inhibitor of histamine binding and is correspondingly more antiproliferative than dec-DPPE. The results support a relationship between antagonism of histamine and growth inhibition but argue against an association between the antiproliferative effects of DPPE and dec-DPPE and inhibition of PKC. The findings for DPPE suggest that platelet response to PMA by diphenylmethane-type AEBS-ligands, may be mediated, at least in part, by mechanisms other than activation of protein kinase C-dependent phosphorylation.

INTRODUCTION

Recent investigations have focused on the study of antiproliferative agents which bind to the microsomal AEBS, a distinct site so-named because it was first identified as a result of binding studies with [³H]tamoxifen (1). The mechanism of the antiproliferative effect of AEBS-ligands is uncertain, although nonestrogen receptor-mediated interactions with calmodulin (2), calcium channels (3), binding sites for tumor-promoting phorbol esters (4), and novel (non-H₁, non-H₂) histamine binding sites (5) have been reported.

A diphenylmethane-derivative AEBS-ligand, DPPE, like tamoxifen, is antiproliferative in vitro (6) and binds with high affinity to AEBS (Kᵢ ~ 5 x 10⁻⁹ M) (7). Unlike tamoxifen, DPPE does not interact with calmodulin (8) or bind to the estrogen receptor (9). Although it is a weak H₁ antagonist (9, 10), DPPE competes for [³H]histamine binding in brain membranes at an AEBS-related site distinct from H₁ and H₂ (11) with an affinity significantly higher than that of traditional H₁ and H₂ antagonists (5). In the same preparation, DPPE also competes for [³H]verapamil binding, but with an affinity higher than that with which verapamil competes for [³H]DPPE and [³H]histamine binding, suggesting distinct but interacting sites near calcium channels for DPPE/histamine and verapamil (5).

The in vitro antiproliferative effect of DPPE does not appear to be mediated through verapamil-type calcium channel antagonism (5), but correlates with its affinity for [³H]histamine binding (5) and is significantly reversed by two amino acids involved in histamine metabolism, L-histidine and L-methionine (5). The findings are compatible with the existence of a growth promoting non-H₁, non-H₂ histamine receptor complex, related to the DPPE/AEBS site, close to calcium channels, but distinct from the verapamil site.

An alternative explanation for the antiproliferative effect of DPPE derives from observations that some AEBS ligands, including tamoxifen and phenothiazines inhibit binding of tumor-promoting phorbol esters and/or protein kinase C action (4, 12). However, decreased inhibition of protein kinase C by these compounds may occur in the presence of increased phospholipid concentration, suggesting an indirect effect (12). Protein kinase C, a ubiquitous calcium and phospholipid-dependent phosphorylating enzyme (13), mediates many important biological processes, including the regulation of hormone function (14) and of growth factors (15). The natural ligand, 1,2-diacylglycerol (16), and esters such as PMA bind to protein kinase C (17). The tumor-promoting properties of phorbol esters are thought to result from their prolonged activation of the enzyme, possibly by the inhibition of its dissociation from plasma membrane to cytosol (18).

In the present study, we have tested the interaction of DPPE and two other diphenylmethane derivatives with protein kinase C. dec-DPPE was synthesized with the intention that the added acyl chain would give greater antiprotein kinase C activity (17). BPD is a decanoyl-diphenylmethane derivative that lacks the alkylaminoethoxy side chain necessary for binding to the DPPE/AEBS site (19). We assessed the ability of DPPE and its analogues to inhibit PMA-induced platelet aggregation, a pathway involving protein kinase C activation (20). The compounds were also tested for inhibition of phosphorylation of...
p47 in platelets, a consequence of protein kinase C activation by PMA (21), and for competition in radioligand binding assays versus [3H]PDBu in platelets, [3H]histamine in rat cortex membranes, and [3H]DPPE in rat liver microsomes. Finally, the agents were assessed for antiproliferative effects against MCF-7 cells in vitro.

MATERIALS AND METHODS

Chemicals

DPPE was synthesized as described previously (7). dec-DPPE was synthesized from 4-hydroxy-DPPE and decanoyl chloride (NuChek Prep. Inc., Elysian, MN). 4-hydroxy-DPPE was synthesized from cis(4-hydroxyphenyl)methane (Aldrich Chemical Co., Milwaukee, WI) and 2-diethylaminoethylchloride-HCl (Aldrich), with isolation of the mono- from the diaduct by silica gel column chromatography. 4-Benzylphenyl decanoate was synthesized from 4-hydroxydiphenylethane (Aldrich) and decanoyl chloride. The chemical structure of all products was verified by nuclear magnetic resonance and mass spectrometry. Solubility studies demonstrated that DPPE is soluble in water, DMSO, and DMF. dec-DPPE is a clear oily solution, miscible in DMSO and DMF, while BPD is insoluble in water but soluble in DMF and DMSO. For binding and growth assays, compounds were dissolved in DMF to a final concentration of 0.1%. For platelet aggregation and phosphorylation studies, they were dissolved in DMSO to a final concentration of 0.5%.

PMA was purchased from LC Services Corp., Worburn, MA.

Isotopes

[3H]DPPE was synthesized by New England Nuclear (Boston, MA). This ring-labeled compound is >98% pure as judged by radiochromatography, 99% pure radiochemically (high-performance liquid chromatography), and is of high specific activity (35.6 Ci/mmol). [3H]-Histamine-2 HCl (48.1 Ci/mmol) and [3H]PDBu (15.8 Ci/mmol) were purchased from New England Nuclear. [32P]Orthophosphate (285 Ci/mg) was purchased from ICN, Montreal, Quebec, Canada.

Preparation of Microsomes and Membranes for Binding Studies

Rat liver microsomal fractions were prepared from adult male Sprague-Dawley rats (7). Platelet microsomes were prepared as described previously (5).

Radioligand Binding Assays

[3H]DPPE Binding in Rat Liver Microsomes. Microsomal fractions were incubated for 5 min at 20°C with 1.5 x 10^{-9} M [3H]DPPE and increasing duplicate concentrations of unlabeled competitors (DPPE, dec-DPPE, or BPD). Unbound label was removed by centrifugation (800 x g) after incubation with dextran-coated charcoal for 15 min at 4°C, and radioactivity in the supernatant was determined. The amount of label bound in the presence of competitor (Bb) was expressed as a ratio of that in the absence of competitor (B0). Percentage Bb/B0 [3H]DPPE was plotted versus log molar (nM) concentration of competitor.

[3H]DPPE binding assays in platelet microsomes were carried out in a manner identical to that for rat liver microsomes.

[3H]Histamine Binding in Rat Brain. Brain membranes were incubated with 1 x 10^{-4} M [3H]histamine in the presence of labeled and unlabeled competitors at room temperature in the dark for 60 min. The reaction was terminated by centrifugation at 12,000 x g for 15 min at 4°C (5). Radioactivity of pellets was quantitated in HP/b scintillation cocktail (Beckman Instruments, Toronto, Ontario, Canada).

Preparation of Platelets. Human whole blood was collected into plastic syringes and mixed with ACD anticoagulant (final pH 6.5) in a ratio of 1.9 ml ACD:8.1 ml blood. Following centrifugation at 100 x g for 5 min (20°C) the PRP was removed. For aggregation studies, the PRP was spun at 1000 x g for 11 min (20°C). The pellet was resuspended in a volume of Hanks' balanced salt solution (pH 7.4) equal to the PRP volume. A 0.2 volume of ACD was added to the mixture. Following centrifugation at 1000 x g for 11 min (20°C) the pellet was resuspended in a volume of Hanks' balanced salt solution equivalent to the original volume of PRP.

For [3H]PDBu binding assays, following 1000 x g centrifugation for 11 min (20°C) as above, the supernatant was removed and the platelet pellet was resuspended in the small amount of remaining plasma, applied to a Sepharose 2B cross-linked column, and eluted with Ca2+-free Tyrode's buffer. The eluate contained approximately 2-4 x 10^6 washed platelets/ml.

[3H]PDBu Binding in Platelets. Duplicate aliquots, each containing 4 x 10^6 freshly prepared, washed, intact platelets, prepared as above, were incubated with [3H]PDBu (15 x 10^{-9} M). Incubation with labeled and unlabeled competitors was carried out at room temperature for 30 min, following which 360-μl aliquots of the incubation mixture were layered over a 250-μl mixture of n-butyl phthalate-diocetyl phthalate (3:1) in microcentrifuge tubes. The tubes were centrifuged at 15,000 x g for 1 min (20°C). The top buffer and then the oil layers were carefully removed using a Pasteur pipet attached to suction. The pellet was dissolved in 0.3 ml 0.2 M NaOH to which was added a further rinse of 0.3 ml 0.2 M NaOH; 500 μl of this mixture were added to 100 μl 1 M HCl and placed in scintillation vials containing 3 ml (50:50) PCS:xylene.

Platelet Aggregometry. Platelet aggregation was measured with a Payton dual-channel aggregometer (Payton Associates, Scarborough, Ontario, Canada) at 37°C. Four hundred-μl aliquots of suspended platelets (2-4 x 10^6 platelets/ml) in siliconized glass cuvetts were warmed for 5-10 min. To the platelet mixture in the aggregometer were added 0.5 mm CaCl_{2} and 485 nM PMA. DDPE, dec-DDPPE, or BPD was added in increasing concentrations to the platelet mixture 30 s before the addition of the PMA. Percentage of aggregation was recorded 3 min after the addition of the agonist (PMA) or the antagonist plus agonist, and compared to the percentage of aggregation produced when buffer alone (minus antagonist) was added 30 s before addition of PMA.

Platelet p47 Phosphorylation. Platelet-rich plasma was incubated for 1 h at 37°C with [32P]orthophosphate (1 mCi/ml), washed, centrifuged at 800 x g for 10 min (20°C), and the pellet resuspended in Tangen-4-(2-hydroxyethyl)-l-piperazineethanesulfonic acid buffer. At time "0," 1-μl aliquots of either DMSO (vehicle) or increasing concentrations of antagonist (DPPE or dec-DPPE) were added to 400-μl aliquots of platelet-rich plasma. Thirty s later, 100-μl aliquots were removed (time 0 control for antagonist or vehicle alone). At time 1 min, 1 μl of PMA (15 or 400 nm) was added to each mixture. Thirty s, 2 or 4 min later, 100-μl aliquots were removed (time, 30 s, 2, or 4 min for PMA plus antagonist or PMA plus vehicle). The 100-μl aliquots were each added to 50-μl aliquots of boiling denaturation solution, boiled for 3 min, and then stored overnight at 20°C. Thawed samples of 40 μl were loaded on a 5-18% polyacrylamide gradient sodium dodecyl sulfate slab gel with a 3% stacking gel and electrophoresed for 4 h at 30 mA/gel constant current. Gels were stained, destained, dried, and autoradiographed. The autoradiographs were visualized to allow identification of the phosphorylated proteins. Individual phosphorylated protein bands were cut from the gel and placed in 7-ml scintillation vials to which was added 1 ml of 80% Protosol (DuPont Chemical Co., Wilmington, DE). The mixture was incubated overnight at 60°C to allow elution of the proteins; 3 ml of scintillation cocktail (49% PCS:49% xylene:2% glacial acetic acid) were added to the vials and radioactivity counted after thorough mixing.

Growth Inhibition Assays. For experiments comparing the antiproliferative effects of DPPE, dec-DPPE, and BPD, 1 x 10^5 MCF-7 human breast cancer cells were seeded into replicate 9.62-cm^2 wells (Linbro, Flow Laboratories, McLean, VA) containing Dulbecco's modified Eagle's medium (Grand Island Biological Co., NY) supplemented with insulin, glucose, and 5%-decomplemented fetal calf serum. After 24 h, the test compounds were added in increasing concentrations (10^{-9}-10^{-6} M) to replicate wells containing medium plus 3.5%-decomplemented fetal calf serum. Control MCF cells were grown in the same
medium containing 0.1% DMF. After 7 days' incubation (37°C), the cells were removed by treatment with Isoton II buffer and counted by Coulter Counter.

Analysis of Binding Data

All binding data were analyzed using the LIGAND computer program (22).

RESULTS

[3H]DPPE Binding in Rat Liver and Platelet Microsomes. DPPE, dec-DPPE, and BPD (Fig. 1) compete for [3H]DPPE binding in rat liver microsomes as shown in Fig. 2. The $K_d$ value for DPPE is $6.5 \pm 2.2$ (SD) x $10^{-8}$ M; the $K_i$ value for dec-DPPE is $7.5 \pm 1.4 \times 10^{-6}$ M; BPD is inactive at concentrations up to $1 \times 10^{-4}$ M. A virtually identical result for DPPE binding is observed in platelet microsomes, where DPPE demonstrates a $K_d$ value of $6.0 \pm 0.1 \times 10^{-8}$ M (data not shown). The estimated number of DPPE/AEBS sites in platelet microsomes is 0.5-2.0 pmol/mg protein, based on dpm comparisons with values previously obtained in rat liver microsomes of similar protein concentration (11).

[3H]Histamine Binding in Rat Cortex Membranes. The affinity of the same ligands for [3H]histamine binding in rat cortex membranes, as shown in Fig. 3, correlates with affinity for [3H]DPPE binding in rat liver microsomes. The $K_i$ value for DPPE is $0.83 \pm 0.62 \times 10^{-6}$ M, that for dec-DPPE is higher at $6.6 \pm 3.5 \times 10^{-6}$ M; BPD is inactive. Affinity of DPPE for [3H]-histamine binding in rat cortex membranes appears to be higher than we reported previously for synaptosomes in the same tissue ($K_i = 4.5 \pm 2.6 \times 10^{-6}$ M) (5).

[3H]PDBu Binding in Platelets. Fig. 4 shows the relative potencies of PMA, DPPE, dec-DPPE, and BPD to compete for [3H]PDBu binding in intact platelets. The $K_i$ value for PMA is approximately $10 \times 10^{-9}$ M. DPPE, dec-DPPE, and BPD all fail to compete for [3H]PDBu binding at concentrations up to $1 \times 10^{-4}$ M.

PMA-induced Platelet Aggregation. The inhibitory effects of DPPE, dec-DPPE, and BPD on PMA (485 nM)-induced platelet aggregation are shown in Fig. 5. dec-DPPE is the most potent ($IC_{50} = 18.8 \pm 0.7 \times 10^{-6}$ M) followed by DPPE (31.2 $\pm 2.4 \times 10^{-6}$ M). BPD is essentially inactive, the curve plateauing at 20% inhibition at concentrations between 50 and $100 \times 10^{-6}$ M.

Inhibition of Phosphorylation of p47 in Platelets. At a concentration of 400 nM PMA, the $IC_{50}$ value for DPPE is $>500 \mu M$; that for dec-DPPE is $200 \mu M$ (Fig. 6A). When the concentration of PMA is reduced to 15 nM, DPPE is still inactive as an inhibitor while the $IC_{50}$ value for dec-DPPE is reduced to 80 $\mu M$ (Fig. 6B).

Antiproliferative Studies. A comparison of the antiproliferative and cytotoxic effects of DPPE and its two analogues on MCF-7 cells (7 days' incubation) is shown in Fig. 7. For DPPE, the $IC_{50}$ value for growth inhibition is $4.5 \pm 0.5 \times 10^{-6}$ M; that for dec-DPPE is $1.5 \pm 0.4 \times 10^{-5}$ M. BPD is ineffective at all
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Fig. 5. Inhibition of platelet aggregation induced by 485 nM PMA in the presence of increasing concentrations of DPPE (○), dec-DPPE (ÇÃO), and BPD (■). Bars, ± SD; N = 3; CONC, concentration.

Fig. 6. A, inhibition of phosphorylation of platelet p47 induced by 400 nM PMA in the presence of increasing concentrations of DPPE (○, 30 s; ◼, 4 min) and dec-DPPE (○, 30 s; ◼, 4 min). B, same experiment using 15 nM PMA in the presence of increasing concentrations of DPPE (○, 30 s; ◼, 2 min) and dec-DPPE (○, 30 s; ◼, 2 min). For dec-DPPE (A) bars, ± SD; N = 2.

Fig. 7. Dose response for growth inhibition of MCF-7 cells after 7 days' exposure to increasing concentrations of DPPE (○), dec-DPPE (○), and BPD (■). For DPPE, cytotoxicity occurred at concentrations >1 × 10^{-5} M; for dec-DPPE, at concentrations >5 × 10^{-5} M. Bars, ± SD; N = 3; CONC, concentration; VS, versus.

concentrations tested. Cytotoxic effects occur at concentrations >1 × 10^{-5} M for DPPE and >5 × 10^{-5} M for dec-DPPE.

DISCUSSION

Within the limitation imposed by extrapolating from data using different systems, the present studies provide evidence to dissociate the antiproliferative effects of diphenylmethane-derivative AEBS-ligands from potency to inhibit protein kinase C action: (a) DPPE, which is antiproliferative, is a very weak antagonist of protein kinase C, as assessed by its inability to significantly inhibit PMA-mediated phosphorylation of p47 in intact platelets, except at very high concentrations. However, despite its lack of effect on protein kinase C, DPPE inhibits PMA-induced platelet aggregation with an IC_{50} in the micromolar range; and (b) dec-DPPE does inhibit protein kinase C, as demonstrated by its greater antagonism of p47 phosphorylation; while also more potent in inhibiting PMA-induced aggregation, it is less potent than DPPE in inhibiting histamine binding and as an antiproliferative agent. The anti-PMA effects of DPPE and dec-DPPE clearly are not related to processes mediated through their inhibition of its binding, nor does there appear to be a good correlation between IC_{50} for inhibition of phosphorylation of p47 and inhibition of platelet aggregation. Alternatively, if PMA-induced platelet aggregation requires only a portion of p47 phosphorylation (e.g., 10–30%), then much higher concentrations of protein kinase C antagonists would be needed for 50% inhibition of p47 phosphorylation than for 50% inhibition of platelet aggregation. The lack of effect of BPD to antagonize PMA action as well as growth suggests that although the two activities are not apparently related, binding to a DPPE/histamine site may be necessary for both.

While differences in metabolic fate between DPPE and dec-DPPE cannot be ruled out, our results suggest that, for diphenylmethane-derivative AEBS ligands, antagonism of histamine binding correlates best with antiproliferative potency. In this regard, it is of interest that PMA induces histidine and ornithine decarboxylase (23). Furthermore, thapsigargin, a tumor promoter structurally related to PMA (24), induces histidine but not ornithine decarboxylase, and does not bind to protein kinase C (25). Our present results, and those with thapsigargin, suggest further studies to test the concept that stimulation of histidine decarboxylase activity may be more critical to promotion of tumor growth than is activation of protein kinase C. Finally, antagonism by DPPE of PMA-induced platelet aggregation occurs in the absence of significant inhibition of protein kinase C-mediated phosphorylation. Thus, activation of histidine decarboxylase might explain, in part, the effects of PMA on platelets.

REFERENCES

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