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INHIBITION OF PROTEIN KINASE C BY CALPHOSTIN C IS LIGHT-DEPENDENT

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Summary. Calphostin C, a secondary metabolite of the fungus *Cladosporium cladosporioides*, inhibits protein kinase C by competing at the binding site for diacylglycerol and phorbol esters. Calphostin C is a polycyclic hydrocarbon with strong absorbance in the visible and ultraviolet ranges. In characterizing the activity of this compound, we unexpectedly found that the inhibition of [^{3}H]phorbol dibutyrate binding was dependent on exposure to light. Ordinary fluorescent light was sufficient for full activation. The inhibition of protein kinase C activity in cell-free systems and intact cells also required light. Light-dependent cytotoxicity was seen at concentrations about 5-fold higher than those inhibiting protein kinase C.

Protein kinase C is an important regulator of cellular function (1). The activity of PKC is dependent on intracellular calcium and diacylglycerol, both of which are regulated by phosphatidylinositol hydrolysis. The enzyme is also stimulated by several natural products that act through the diacylglycerol site, including phorbol esters, teleocidin, and bryostatin.

Specific inhibitors of PKC would be very useful as pharmacological tools and possibly also as drugs. Although staurosporine and other indolocarbazoles are potent inhibitors of PKC, these compounds also inhibit other protein kinases, including cyclic AMP-dependent protein kinase (2). The poor specificity of the indolocarbazoles stems from the fact that they act at the catalytic site, a site that is conserved throughout the protein kinase family of enzymes.

In contrast to the catalytic site, the phorbol/diacylglycerol site is unique to PKC. An inhibitor that acted at this site would therefore be predicted to be highly selective for PKC compared to other kinases. In agreement with this prediction, the first such inhibitor to be reported, calphostin C, showed potent

Abbreviations: EGF, epidermal growth factor; PDB, phorbol dibutyrate; PKC, protein kinase C; PMA, phorbol myristate acetate.

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inhibition of PKC (IC_{50} 50 nM) with negligible activity at other protein kinases (3). Calphostin C is a deep red perylenequinone metabolite of the fungus *Cladosporium cladosporioides* (Figure 1), and is a member of a family of deeply colored perylenequinones that are produced by various fungal plant pathogens (4). Other perylenequinones from this family are known to show strong phototoxicity to plant, animal, and bacterial cells (5,6). In the present study, we show that the inhibition of PKC by calphostin C also requires light.

MATERIALS AND METHODS

BSA, bacitracin, PDB, and polyethylenimine were from Sigma Chemicals. [^{3}H]PDB (19.1 Ci/mmol), [^{3}H]arachidonic acid (180-240 Ci/mol), and [^{125}I]EGF (150-200 μCi/μg) were from Du Pont New England Nuclear. Calphostin C was isolated as described (7). The A431 cell line was from the American Type Culture Collection and the C3H10T1/2 (clone 8) cell line was from the late Dr. Charles Heidelberger (University of Southern California Comprehensive Cancer Center). Rats were from Harlan Sprague-Dawley. Sources of materials for the assay of PKC activity were as previously described (3).

[^{3}H]PDB binding assay: Forebrains from Sprague-Dawley rats were removed and disrupted by Polytron in 10 volumes of ice-cold 50 mM Tris-HCl pH 8.3 (pH 7.7 at 25 °C) with 1 mM $CaCl_2$ (assay buffer). The homogenate was centrifuged for 12 min at 44,000 xg, and the pellet resuspended in the same buffer. After two additional cycles of centrifugation/resuspension, the pellet was resuspended and stored at -80 °C. Tissue suspensions were disrupted with a Polytron after thawing. The order of additions for [^{3}H]PDB binding was 195 μl of Tris-$CaCl_2$, 100 μl [^{3}H]PDB in assay buffer (final concentration 2 nM), test agent (calphostin C or unlabeled PDB) in 5 μl DMSO, and rat brain membranes (10-15 μg protein) in 200 μl assay buffer. Control incubations contained an equal concentration of DMSO. Nonspecific binding was defined by addition of 5 μM unlabeled PDB. Incubations were carried out for 60 min at room temperature in Beckman deep-well microtiter plates. "Light" incubations were carried out on an open benchtop under ordinary cool-white fluorescent lighting; "dark" incubations were carried out in a darkroom under a red safelight, and samples were kept covered with aluminum foil during the short interval between removal from the darkroom and filtration. Assays were terminated by filtration on a Brandel 48R cell harvester through GF/B filter sheets that had been presoaked for 90 min in 1 g/l BSA + 0.14 g/l bacitracin + 3 g/l polyethylenimine in 50 mM Tris-HCl pH 7.7. Filters were transferred to 7-ml minivials, left overnight in 5 ml of Beckman Ready-Protein Plus, shaken, and counted in a Taurus scintillation counter.

Protein kinase C assay: Protein kinase C was partially purified from rat brain via DE-52 and phenylsepharose chromatography as described (8). Enzyme activity was assayed in 250 μl of 20 mM Tris-HCl pH 7.5 with 10 mM magnesium acetate, 50 μg histone IIIS, 20 μg phosphatidylserine, 0.88 μg diolein, 50 μM $CaCl_2$, 5 μM [γ-^{32}P]ATP (125,000 cpm), and 1 μg partially purified protein kinase C according to the method of Kikkawa *et al.* (8).

Down-regulation of the EGF receptor by PDB: A431 human epidermoid carcinoma cells were grown to confluency in 24-well tissue culture plates using Dulbecco's Modified Eagle's Medium supplemented with 10% heat-inactivated fetal bovine serum. After reaching confluence, cells were treated with calphostin C for 3 hr in phosphate-buffered saline with 0.1% BSA (incubation medium) at 23 °C in the presence and absence of fluorescent room light. PDB was added to the cells to give a final concentration of 50 nM and the cells were transferred to a humidified 5% CO_2 incubator kept at 37 °C. After 2 hr the medium was removed and the cells washed with ice-cold incubation medium. The cells were then incubated with 1 ml of incubation medium

containing 0.1 ng/ml of [^{125}I]EGF for 2 hr at 4 °C. The binding medium was removed and the cells washed with ice-cold incubation medium. Radioactivity was solubilized in 1 N NaOH and quantitated by gamma counting.

Arachidonic acid release: C3H10T1/2 cells were plated into 12-well culture dishes at 5-10 x 10^4 cells/cm^2. Twenty-four hr after plating, fresh medium containing 1.28 μCi of [^{3}H]arachidonic acid in 1 ml was added. After 3 days, the labeled cells were washed once with growth medium (Eagle's basal medium with 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum) and incubated in 500 μl of growth medium with test agent (PMA and/or calphostin C) or vehicle. After 2 hr, an additional 500 μl of growth medium with test agent or vehicle was added. The incubation was continued for 2 hr, after which 500 μl of medium was removed for scintillation counting. The incubation with test agent was carried out in an unmodified incubator (dark) or in an incubator with an 8-watt fluorescent light source located 15 cm above the culture dishes.

RESULTS

During studies designed to confirm and extend the initial results of Kobayashi *et al.* (3), small discrepancies from the published results were observed in the affinity of calphostin C for inhibition of [^{3}H]PDB binding. Because calphostin C is deeply colored (Fig. 1), we speculated that the differences might have been due to photodegradation of calphostin C. To test this possibility, experiments were carried out in the dark. Unexpectedly, calphostin C showed little or no ability to inhibit [^{3}H]PDB binding in the absence of light (Fig. 2). Inhibition of PKC enzyme activity was also dependent on light (Fig. 3). Subsequent to this observation, a review of the literature revealed a sizable body of studies dating back to 1957 (5,6) on photodynamic activity of perylenequinones (reviewed in ref. 4).

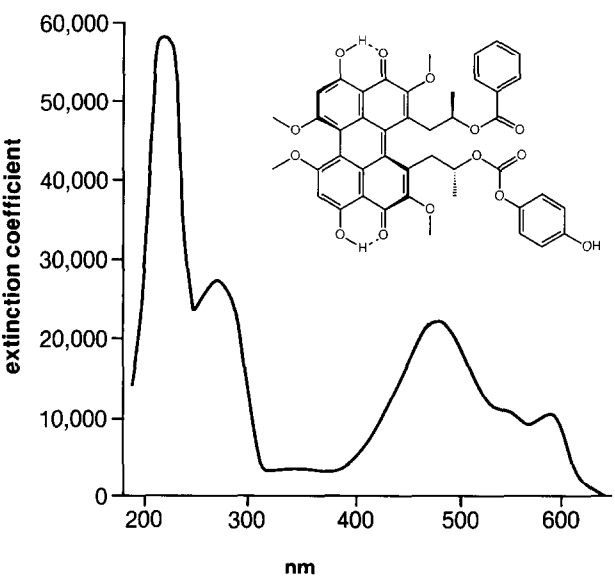


Figure 1. **Structure and UV/Vis absorbance spectrum of calphostin C.** The absorbance spectrum was determined in methanol.

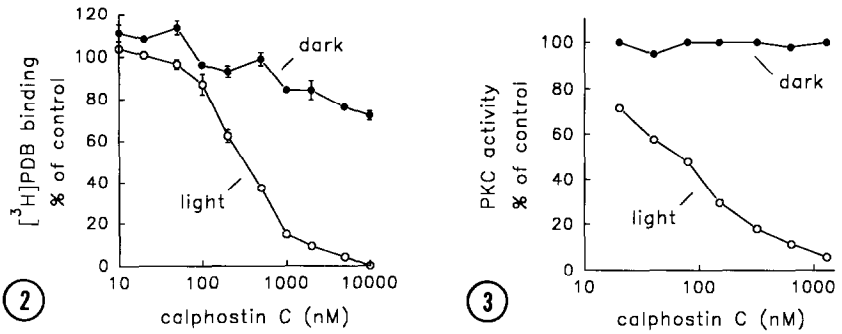


Figure 2. **Light dependence of the inhibition of [^{3}H]PDB binding by calphostin C.** Incubation of 2 nM [^{3}H]PDB with rat brain membranes was carried out under ordinary fluorescent light or in a darkroom as described in **MATERIALS AND METHODS**. Values are specific binding. Inhibition of [^{3}H]PDB binding by calphostin C in the dark varied considerably, with some experiments (this figure and Fig. 4B) showing little inhibition and others (Fig. 4A) exhibiting weak but significant inhibition. The differences in inhibition in the dark may be related to variations in solubility of calphostin C or to differences in the amount of exposure to light just prior to filtration.

Figure 3. **Light dependence of the inhibition of protein kinase C activity by calphostin C.** Diolein-stimulated protein kinase C activity was measured as described in **MATERIALS AND METHODS**.

Additional experiments were carried out to characterize the photodynamic activity of calphostin C. Preincubation of membranes with calphostin C in the presence of light, followed by incubation with [^{3}H]PDB in the dark, resulted in inhibition of binding (Fig. 4). However, preincubation of calphostin C with light and [^{3}H]PDB in the absence of membranes, followed by incubation with membranes in the dark, did not produce any inhibition (Fig. 4). The results in Fig. 4 imply that photoactivation of calphostin C generates a short-lived species that reacts with PKC or another membrane component, resulting in permanent inactivation of PDB binding. The lack of inhibition by calphostin

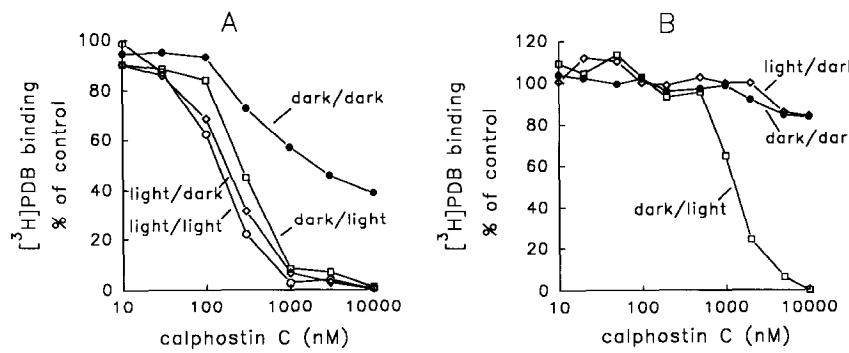
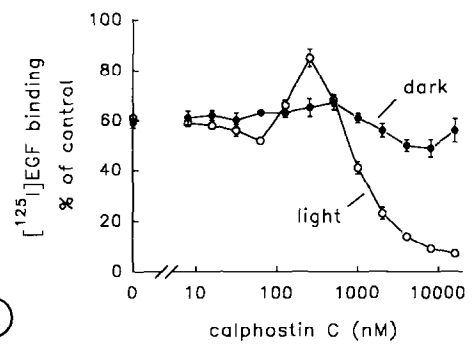
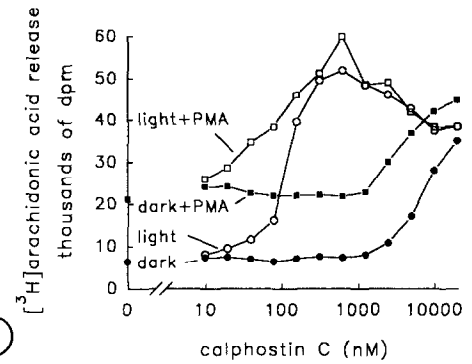


Figure 4. **Effects of preincubation of calphostin C with membranes (A) or [^{3}H]PDB (B).** Calphostin C was preincubated for 60 min in the light or dark, after which the remaining component of the assay ([^{3}H]PDB in A, membranes in B) was added and the incubation continued for an additional 60 min in the light or dark. For instance, "light/dark" denotes preincubation in the light followed by a binding incubation in the dark.



(5)



(6)

Figure 5. **Effect of calphostin C on down-regulation of [^{125}I]EGF binding by 50 nM PDB in A431 cells.** [^{125}I]EGF binding is expressed as a percentage of control specific binding in the absence of PDB.

Figure 6. **Effect of calphostin C on arachidonic acid release in C3H10T1/2 cells.** Cells prelabeled with [^{3}H]arachidonic acid were incubated for 4 hr in the presence and absence of 100 ng/ml PMA and light.

C exposed to light in the absence of membranes also eliminates the possibility that photoisomerization of the perylene ring twist (4,9) can account for the light-dependence.

Phosphorylation of the EGF receptor by PKC lowers the affinity of the receptor for EGF, resulting in a decrease in [^{125}I]EGF binding (10). This assay was used to measure functional antagonism of PKC by calphostin C. In the presence of 50 nM PDB, calphostin C showed a bell-shaped curve, reversing the inhibition by PDB at lower concentrations and inhibiting [^{125}I]EGF binding at higher concentrations (Fig. 5). The inhibition of [^{125}I]EGF binding seen at higher concentrations was due to cytotoxicity during the preincubation, since calphostin C did not directly inhibit [^{125}I]EGF binding. Both the reversal of the PDB inhibition and the cytotoxicity were light-dependent (Fig. 5). Since the reversal of PDB inhibition occurred at about 5-fold lower concentrations than the cytotoxicity, these results suggest some degree of specificity in the interaction between calphostin C and PKC.

Phorbol esters increase arachidonic acid release in various cell lines (11). When tested in C3H10T1/2 cells, calphostin C by itself caused a light-dependent release of arachidonic acid (Fig. 6), which appeared to be additive with the release caused by PMA. These results are consistent with a light-dependent cytotoxic action of calphostin C.

DISCUSSION

The photodynamic activity of perylenequinones has been known since at least 1957 (5,6). The present study describes the fortuitous rediscovery of this photodynamic activity in calphostin C, a new member of this class of compounds.

In the presence of light, other perylenequinones are known to react with molecular oxygen, generating singlet oxygen (12) and forming various

endoperoxides (13,14). Membrane destruction resulting from lipid peroxidation by singlet oxygen is thought to mediate the killing of plant cells by the perylenequinone cercosporin (4,15). Although these mechanisms undoubtedly account for the toxicity to mammalian cells of calphostin C, it is not clear whether they also account for the photoinhibition of PKC. The inhibition of PKC functional activity at concentrations that do not cause cytotoxicity (Fig. 5) suggests a more specific interaction between calphostin C and PKC. In agreement with this idea, calphostin C in the presence of light had little inhibitory activity ($IC_{50} \geq 10$ μM) at other membrane receptors, including dopamine D_2, bradykinin, and EGF receptors. Also, in preliminary experiments, antioxidants and singlet oxygen quenchers did not prevent the inhibition of [^{3}H]PDB binding by calphostin C. A more specific mechanism might involve photoaffinity labeling of the phorbol site of PKC; however, unlike calphostin C, most photoaffinity ligands have good affinity in the dark. Alternatively, an endoperoxide of calphostin C could react irreversibly with PKC. Additional studies to distinguish these possibilities might include experiments under oxygen-free conditions and the use of radiolabeled calphostin C to search for covalent labeling.

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