

Irreversible oxidative inactivation of protein kinase C by photosensitive inhibitor calphostin C

Rayudu Gopalakrishna, Zhen Hai Chen and Usha Gundimeda

Department of Pharmacology and Nutrition, School of Medicine, University of Southern California, Los Angeles, CA 90033, USA

Received 26 September 1992; revised version received 26 October 1992

Isolated protein kinase C (PKC) was irreversibly inactivated by a brief (min) incubation with calphostin C in the presence of light. This inactivation required Ca^{2+} either in a millimolar range in the absence of lipid activators or in a submicromolar range in the presence of lipid activators. In addition, an oxygen atmosphere was required suggesting the involvement of oxidation(s) in this inactivation process. Furthermore, PKC inactivation might involve a site-specific oxidative modification of the enzyme at the Ca^{2+} -induced hydrophobic region. Physical quenchers of singlet oxygen such as lycopene, β -carotene, and α -tocopherol all reduced the calphostin C-induced inactivation of PKC. In intact cells treated with calphostin C, the inactivation of PKC was rapid in the membrane fraction compared to cytosol. This intracellular PKC inactivation was also found to be irreversible. Therefore, calphostin C can bring prolonged effects for several hours in cells treated for a short time. Taken together these results suggest that the calphostin C-mediated inactivation of PKC involves a site-specific and a 'cage' type oxidative modification of PKC.

Protein kinase C; Reactive oxygen species, Singlet oxygen; Calphostin C; Oxidative modification; Ca^{2+} -induced hydrophobic region

1. INTRODUCTION

Protein kinase C (PKC) has been implicated to play a key role in signal transduction and also in tumor promotion [1–3]. Terpenoid tumor promoters such as phorbol esters induce not only the activation of PKC by direct binding to the enzyme but also the downregulation of PKC by an indirect mechanism [1–5]. Oxidant tumor promoters either activate or inactivate PKC depending on the extent of oxidative modification [6–8]. A selective oxidative modification of the regulatory domain results in a Ca^{2+} /lipid-independent activation of the kinase with a loss of phorbol ester binding [6]. Alternately, a selective oxidative modification of the kinase domain results in the generation of a modified form of PKC which exhibits only phorbol ester binding [8]. Some oxidants such as H_2O_2 induce irreversible oxidative modification [6], whereas others such as *m*-periodate induce reversible oxidative modification [8]. Furthermore, PKC (the phorbol ester receptor) was shown to be regulated by lipid peroxidation [9], redox cycling quinones [10], and oxidant-generating systems [11].

Since reactive oxygen species have been implicated in various disease states [12], PKC may be oxidatively regulated under these pathophysiological conditions.

Agents that selectively inhibit PKC are desirable tools to assess the role of PKC in various cellular functions and also as therapeutic agents. Furthermore, understanding the mechanism of action of such agents is important to design and develop more specific inhibitors. There have been two types of PKC inhibitors reported: one binds to the regulatory domain and the other binds to the catalytic domain [13–15]. Calphostin C is a perylenequinone metabolite isolated from the fungus *Cladosporium cladosporioides* [16]. It is one of the specific inhibitors of PKC and it inhibits both phorbol ester binding and phosphotransferase activity of PKC presumably by binding to the regulatory domain [16]. Moreover, the inhibition of PKC by calphostin C is light dependent [17]. Although calphostin C has been extensively employed as a pharmacological tool, the mechanism of its action is not elucidated. It is not known whether the light-reaction products of calphostin C induced a reversible inhibition by binding to PKC. Alternately, an irreversible inactivation may be induced by either oxidative modification of the amino acid residues within PKC, or by a covalent interaction of calphostin C with the enzyme. Since we have been involved in understanding the regulation of PKC by oxidations, we have attempted to determine the possible involvement of oxidations in calphostin C-mediated inhibitions of PKC. Our results suggested that calphostin C induced a site-specific oxidative modification of PKC at the Ca^{2+} -induced hydrophobic region. This resulted

Correspondence address. R. Gopalakrishna, Department of Pharmacology and Nutrition, School of Medicine, University of Southern California, 2025 Zonal Avenue, Los Angeles, CA 90033, USA. Fax: (1) (213) 221-1235.

Abbreviations. PKC, protein kinase C; EGTA, ethylene glycol bis(β -aminoethylether)-*N,N'*-tetraacetic acid; PDBu, phorbol 12,13-dibutyrate; TPA, 12-*O*-tetradecanoyl-phorbol 13-acetate.

in an irreversible inactivation of PKC both in the isolated form and in intact cells.

2. MATERIALS AND METHODS

2.1 Materials

Calphostin C was purchased from the LC Services Corporation. β -Carotene and DL- α -tocopherol were obtained from Fluka. Lycopene and *N*-acetylcysteine were supplied by the Sigma Chemical Company. PKC from rat brain was purified to apparent homogeneity as described previously [18]. In some experiments partially purified PKC (approximately 600 units/mg protein) was used. M-kinase was derived from native PKC by limited proteolysis using calpain II as described previously [6].

2.2 PKC treatment with calphostin C

Although PKC at various stages of purification exhibited sensitivity to calphostin C-mediated inhibition, PKC in freshly purified preparations was completely inhibited by calphostin C. Purified PKC (2 units) was incubated with calphostin C along with various factors in a total volume of 0.5 ml in polystyrene tubes at room temperature under ordinary fluorescent light for 3 min. Then, 25 μ l of bovine serum albumin solution (10 mg/ml) and 25 μ l of 0.1 M EDTA were added. Calphostin C and other low-molecular-weight compounds were removed from the treated PKC samples by subjecting them to a 'centrifuge column technique' [19].

2.3. Isolation of PKC from calphostin-treated cells

The cytosolic and detergent-solubilized membrane fractions were prepared from control and calphostin C-treated cells as described previously [18]. These fractions (2.5 ml) were applied to 0.5-ml DEAE-cellulose (DE-52) columns previously equilibrated with buffer A (20 mM Tris-HCl, pH 7.5/1 mM EDTA/0.1 mM DTT). After washing the column with 2 ml of buffer A, the bound PKC was eluted with 1.25 ml of 0.1 M NaCl in buffer A.

2.4 PKC assay and PDBu binding

Both PKC phosphotransferase activity and phorbol ester binding were determined by using a multiwell plate filtration assay utilizing the MultiScreen system (Millipore) as described elsewhere [20]. Briefly, PKC reaction samples in a total volume of 125 μ l were incubated in 96-well plates with fitted filtration discs made of cellulose acetate membranes (HA type). The histone H1 was precipitated and filtered with 10% TCA and the radioactivity associated with the filters was counted. Similarly, for the determination of PKC-associated phorbol ester binding, both incubations and filtrations were carried out in multiwell plates fitted with filtration discs. The samples were incubated with [3 H]phorbol 12,13-dibutyrate (PDBu) in the microwells, the ligand-bound PKC was adsorbed onto DEAE-Sephadex beads, and the beads then were filtered and washed in the same multiwells. The radioactivity associated with the DEAE-Sephadex beads retained on the filter was counted. PKC activity was expressed as units, where one unit of enzyme transfers 1 nmol of phosphate to histone H1 per min at 30°C.

3. RESULTS

In previous studies, calphostin C was added directly to the kinase assay or to the PDBu binding incubation to determine its effect on PKC [15,16]. Since the PKC assay mixture contains various other components, it is very difficult to determine the factors that influence the action of calphostin C. Furthermore, it is also difficult to distinguish whether calphostin C is inducing an inhibition or a permanent inactivation of PKC. Therefore,

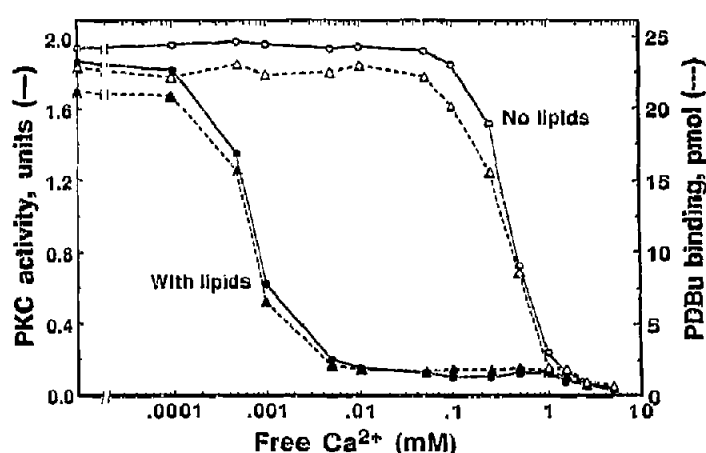


Fig. 1. Ca^{2+} requirement for inactivation of PKC by calphostin C. Purified PKC samples were treated with calphostin C (1 μ M) in the presence of light. The indicated concentrations of free Ca^{2+} were maintained by using EGTA- CaCl_2 buffer. To one set of samples (open symbols) no lipid activators were added, whereas to the other set of samples (filled symbols) 10 μ g of phosphatidylserine and 0.4 μ g of diolefin were added. In the samples incubated with lipids there was a need to add ATP (0.1 mM) and Mg^{2+} (8 mM) to protect the enzyme. Once the enzyme was incubated with Ca^{2+} and lipids, it was converted to a lipoprotein form which no longer required further addition of Ca^{2+} and lipids for expressing the kinase activity. This lipid-treated PKC when assayed in the absence of added Ca^{2+} and lipids, exhibited nearly 60 to 80% of the optimal activity obtained by incubating with Ca^{2+} and lipids. Hence the total activity observed with Ca^{2+} and lipids was presented without subtracting the Ca^{2+} /lipid-independent basal activity. After separation of calphostin C from the treated PKC samples, both kinase activity and PDBu binding of PKC were determined by multiwell filtration assays. The values represent mean of three replicate estimations.

to understand the mechanism of the calphostin C effect on PKC, initially PKC was treated with calphostin C under defined conditions, and then calphostin C was removed from the treated PKC sample. Thus, at a later step during the determination of kinase activity and PDBu binding, calphostin C was no longer present.

3.1. Ca^{2+} requirement for PKC modification by calphostin C

Initially PKC was incubated under laboratory fluorescent light with calphostin C (0.01–1 μ M) for 3 min in the presence of 1 mM EGTA. After removing the calphostin C, the treated PKC samples were analyzed, and there was no decrease in either kinase activity or PDBu binding. On the contrary, when PKC was incubated with calphostin C in the presence of Ca^{2+} , PKC lost irreversibly both kinase activity and PDBu binding. The optimal concentration of Ca^{2+} required was in the range of 1–1.5 mM (Fig. 1). However, in the presence of phosphatidylserine and diolefin the Ca^{2+} required for calphostin C-mediated inactivation was decreased to as low as 1 μ M. Nonetheless, no inactivation was noted with these lipids in the presence of 1 mM EGTA. Similarly, TPA along with phosphatidylserine also de-

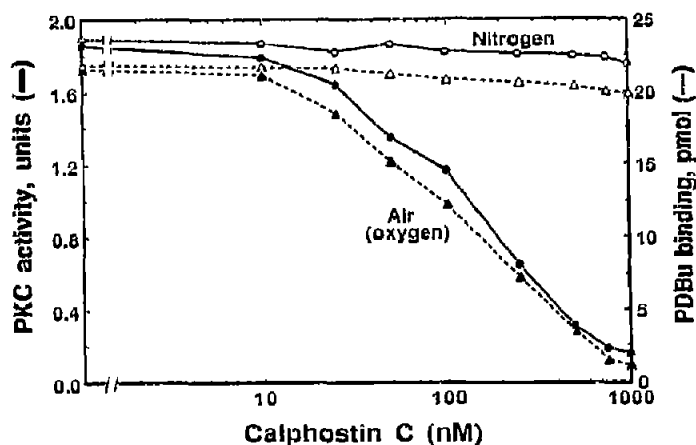


Fig. 2 The oxygen requirement for PKC inactivation by calphostin C. These PKC modifications were carried out in the absence of lipids in microfuge tubes fitted with rubber stoppers. To two sets of PKC samples, indicated concentrations of calphostin C were added along with 1 mM EGTA. One set of samples (open symbols) was left in normal atmospheric air, whereas in the other set of samples (closed symbols) air was displaced with nitrogen gas. To initiate PKC modification, CaCl_2 was injected to both sets of samples to obtain 1 mM free Ca^{2+} . Then the samples were incubated for 3 min in the presence of light and EGTA was added to a final concentration of 5 mM. Both PKC activity and PDBu binding were determined in the treated samples.

created the requirement of Ca^{2+} for PKC inactivation. PKC activity did not recover by subjecting the calphostin C-inactivated PKC to extensive dialysis, to hydrophobic chromatography, or to DEAE-cellulose chromatography. Conceivably, the observed decrease in

PKC activity was due to the irreversible modification of PKC induced by calphostin C.

3.2. Oxygen requirement for photo-inactivation

Since the calphostin C-mediated inactivation of PKC was a light-dependent reaction, we tested whether any oxidations were involved in this inactivation. As shown in Fig. 2, although calphostin C inactivated PKC in the presence of normal atmospheric air (presence of oxygen), under a complete nitrogen atmosphere (absence of oxygen) it failed to inactivate PKC even at a higher concentration (1 μM). This suggested that calphostin C-mediated inactivation of PKC involves oxidation(s).

3.3. The requirement of Ca^{2+} -induced accessible hydrophobic site

Based on our previous observations that Ca^{2+} induced the hydrophobic reaction on PKC [21] and also Ca^{2+} was required for calphostin C-mediated PKC inactivation as shown in the present study, we assessed whether the Ca^{2+} -induced hydrophobic site may be required for this inactivation. For this purpose we used two preparations of PKC that exhibited a differential sensitivity to calphostin C-mediated inactivation. PKC in freshly purified preparations from rat brain was inactivated by calphostin C above 95%, whereas the preparation of PKC purified and stored for 3 months at -20°C was inactivated only 50–70% by calphostin C. PKC exhibits two types of binding to phenyl-Sepharose: one is a Ca^{2+} -independent weak hydrophobic binding which requires a high ionic strength (0.5 M NaCl), whereas the later type is a Ca^{2+} -induced binding, which is stronger and

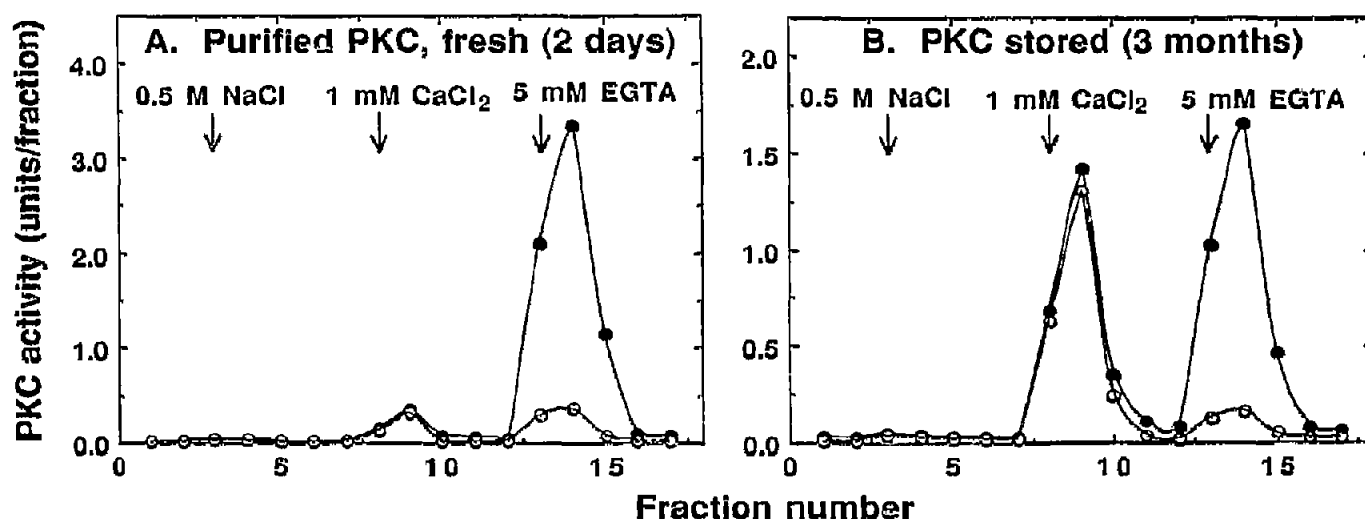


Fig. 3 Effect of calphostin C on the purified PKC that Ca^{2+} -dependently retained on the phenyl-Sepharose column. (A) Purified PKC used within two days after isolation from fresh rat brains. (B) Purified PKC preparation stored for 3 months at -20°C . PKC samples (2 ml) were applied to a 1 ml phenyl-Sepharose column previously equilibrated with buffer A containing 0.5 M NaCl and allowed to Ca^{2+} -independently bind to the resin by washing with the same buffer. Then the column was washed with buffer A containing 1 mM CaCl_2 in place of 1 mM EDTA. Then the Ca^{2+} -dependently bound PKC was eluted with 5 mM EGTA. Fractions of 1 ml were collected, and subdivided into two portions. One set of subfractions were treated with calphostin C. Then PKC activity present in all these subfractions was determined after removing calphostin C. (●—●) PKC activity in subfractions not treated with calphostin C; (○—○) PKC activity in subfractions treated with calphostin C.

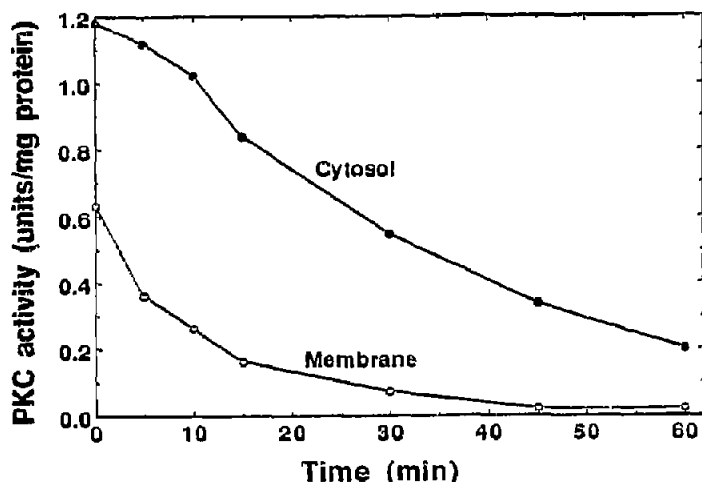


Fig. 4. Inactivation of PKC in intact cells treated with calphostin C. Confluent C6 glioma cells were treated with calphostin C (0.5 μ M) for the indicated time periods. Then both the cytosolic and detergent-solubilized membrane fractions were prepared and PKC activity was determined after isolation by DEAE-cellulose chromatography.

can occur even in a low-ionic-strength buffer (without salt). The Ca^{2+} -induced hydrophobic region on PKC in both fresh and stored preparations was evaluated by Ca^{2+} -dependent retention of PKC on phenyl-Sepharose in a low-ionic-strength buffer. PKC activity present in both preparations was completely bound Ca^{2+} -independently to phenyl-Sepharose at a high-ionic-strength buffer (0.5 M NaCl). Furthermore, most of the (95%) activity of freshly purified PKC was Ca^{2+} -dependently retained on the phenyl-Sepharose when the bound enzyme was washed with a low-ionic-strength buffer containing 1 mM CaCl_2 (Fig. 3A), while with the stored enzyme, only 55% of the activity was retained under these conditions (Fig. 3B). Approximately 40% of the stored PKC, although it had both kinase activity and PDBu binding, did not retain Ca^{2+} -dependently with phenyl-Sepharose when the column was washed with a low-ionic-strength buffer with CaCl_2 . Thus, the Ca^{2+} -independent weak hydrophobic region on PKC was not affected by storage, while a fraction (40%) of the PKC molecules in the stored preparation lost the ability to expose the strong hydrophobic site in the presence of Ca^{2+} . Only the fraction of PKC exhibiting the Ca^{2+} -induced hydrophobic site was inactivated by calphostin C, whereas the fraction of PKC that did not exhibit a Ca^{2+} -induced hydrophobic site was resistant to calphostin C inactivation. Moreover, the calpain-digested PKC (M-kinase) that lacked the Ca^{2+} -induced hydrophobic region was not inactivated by calphostin C.

Some proteins, such as hemoglobin, have been shown to undergo fragmentation upon exposure to oxidants [22]. However, calphostin C-treated PKC showed no decrease in molecular weight as judged by SDS/polyacrylamide electrophoresis. Nonetheless, the inac-

tivated PKC failed to bind phenyl-Sepharose in a low-ionic-strength buffer when it was monitored by immunoblotting. This suggests that the Ca^{2+} -induced hydrophobic region on PKC is required to mediate and is also affected by a calphostin C-induced modification of PKC. Whether there was any formation of covalent adducts of calphostin C with PKC during photoexcitation was evaluated. Since calphostin C is a bright red compound, the visible absorption spectrum of the calphostin C-treated PKC (0.8 mg, 10 nmol) was determined. There was no detectable amount of chromogenic material associated with the calphostin C-treated PKC after it was subjected to a centrifuge column technique. This suggested that if there was any low (nonstoichiometric) amount of calphostin C covalently associated with PKC below the limits of detection (<1 nmol), it was unlikely to be responsible for the observed total loss of PKC activity.

3.4. Possible involvement of singlet oxygen in the site-specific oxidative inactivation of PKC by calphostin C

Since photoexcitation of perylenequinones was shown to generate singlet oxygen [23], the possible involvement of singlet oxygen in PKC inactivation was evaluated. Initially the calphostin C alone was exposed to light and then light-treated calphostin C was added to PKC in the dark. This two-step treatment did not produce any inactivation of PKC. Simultaneous exposure of PKC and calphostin C to light was required to induce the inactivation of PKC. Then, both physical and chemical quenchers of singlet oxygen [24] were tested to inhibit calphostin C-mediated inactivation of PKC. Hydrophobic physical quenchers of singlet oxygen such as lycopene (10 μ M), β -carotene (10 μ M), and α -tocopherol (50 μ M) substantially (80–90%) decreased the inactivation of PKC by calphostin C (Table I). However, hydrophilic chemical quenchers such as *N*-acetylcysteine, 2-mercaptoethanol or dithiothreitol

Table I

Effect of physical and chemical quenchers of singlet oxygen on the calphostin C-mediated inactivation of PKC

Treatment	PKC activity (units)	PDBu binding (pmol)
Control untreated	1.96	24.2
Calphostin C (1 μ M) alone	0.29	3.1
Calphostin C (1 μ M) along with quenchers of singlet oxygen		
Lycopene (10 μ M)	1.74	21.2
β -Carotene (10 μ M)	1.67	20.8
α -Tocopherol (50 μ M)	1.64	19.9
<i>N</i> -Acetylcysteine (5 mM)	0.27	2.8
2-Mercaptoethanol (5 mM)	0.26	2.4
Dithiothreitol (5 mM)	0.24	2.2

These calphostin C-mediated PKC modifications were carried out in the presence of 1 mM free Ca^{2+} and in the absence of lipids.

failed to inhibit the inactivation of PKC by calphostin C even at a very high (5 mM) concentration. The protection offered by carotenoids and tocopherol was unlikely due to the competition of these hydrophobic agents with calphostin C for the binding site on PKC, since the nonspecific hydrophobic agents such as adriamycin (100 μ M), phosphatidylserine and other phospholipids (20 μ g/ml) failed to protect PKC from calphostin C-mediated inactivation.

Calphostin C at a 100–500-fold higher (20–100 μ M) concentration inactivated PKC in a Ca^{2+} -independent manner. This inactivation of PKC at higher concentrations of calphostin C was prevented by water-soluble thiol agents as well as hydrophobic singlet oxygen quenchers. It is possible that at low (nanomolar) concentrations of calphostin C, the amounts of reactive oxygen species formed may be too low to induce the oxidative modification of PKC. Nevertheless, in the presence of Ca^{2+} , calphostin C may bind initially to the Ca^{2+} -induced hydrophobic site. Then the reactive oxygen species generated from this protein-bound calphostin C by a light reaction may selectively modify the oxidation susceptible amino acid residue(s) present within the vicinity. Since such an oxidative modification is a 'cage' type reaction, the externally added thiol agents cannot quench the oxidant formed from the protein-bound ligand. However, the hydrophobic quenchers of singlet oxygen (carotenoids and tocopherol) may bind to a hydrophobic site in the near vicinity of the calphostin C-binding region and thereby have the access to trap reactive oxygen species formed. On the contrary, at higher concentrations (μ M) of calphostin C, the concentration of reactive oxygen species generated may be

high enough to induce nonspecific modifications in the PKC molecule resulting in the inactivation of the enzyme in a manner similar to that observed with H_2O_2 and *N*-chlorosuccinimide [6,7]. Since under these conditions the oxidants are generated externally to the protein, both water-soluble and hydrophobic quenchers could prevent the oxidative modification of PKC.

3.5. Inactivation of PKC in intact cells

Treatment of C6 glioma cells with calphostin C (0.1 and 1 μ M) in the presence of light resulted in a loss of both kinase activity and PDBu binding. This inactivation of PKC was rapid in the membrane fraction compared to cytosol (Fig. 4). Furthermore, prior treatment with TPA (100 nM) to induce membrane association of PKC resulted in a more rapid inactivation of PKC compared to that of cells treated with calphostin C alone (Fig. 5). In all these cases the PKC activity was not recovered by an incubation with 5 mM DTT. We then tested whether the removal of calphostin C from the treated cells could result in the recovery of PKC to an active form. C6 glioma cells were initially treated with calphostin C (0.1 μ M) for 2 h and then the treated cells were washed twice with Hank's balanced salt solution and twice with DMEM medium. The washed cells were kept in fresh medium with no calphostin C. Similarly treated and washed cells were left in a medium with cycloheximide (10 μ g/ml) to prevent protein synthesis. In the cells where protein synthesis was allowed, PKC was returned to the control level within 12 h. However, in the cells where protein synthesis was inhibited by cycloheximide, no recovery of PKC activity was observed (data not shown). This suggested that PKC was inactivated irreversibly in the cells and the PKC protein was not repaired by any intracellular mechanisms operating in the cell.

4. DISCUSSION

The currently characterized oxidative modification of PKC induced by calphostin C is different from our previously revealed PKC modifications induced by oxidant tumor promoters such as H_2O_2 and *m*-periodate [6–8] in at least four aspects. First, the inactivation of PKC induced by calphostin C at nanomolar concentrations is strictly a Ca^{2+} -dependent process, whereas the modifications induced by H_2O_2 and *m*-periodate are not strictly Ca^{2+} -dependent, which however are enhanced by Ca^{2+} . Second, there is no transient formation of an oxidatively activated Ca^{2+} /lipid-independent form of PKC during modification by calphostin C. Third, *m*-periodate and H_2O_2 require higher (μ M to mM) concentrations to induce oxidative modification of PKC. However, calphostin C, by initially binding to the Ca^{2+} -induced hydrophobic site on PKC, and then undergoing photo-oxidation can induce a site-specific modification even at lower (nanomolar) concentrations. Finally, the

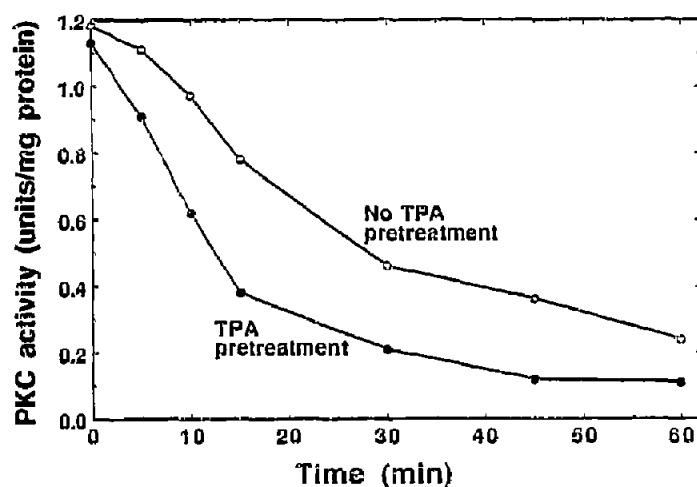


Fig. 5. Effect of TPA pretreatment on the calphostin C-mediated inactivation of PKC in intact cells. Confluent C6 glioma cells were treated initially with TPA (100 nM) for 30 min to induce a cytosol to membrane translocation of PKC. Then both control and TPA-treated cells were incubated with calphostin C (0.5 μ M) in the presence of light for indicated time periods. Then, total PKC (cytosol and membrane) was extracted from the cells with a buffer containing detergent and PKC activity was determined as described in section 2.

modifications of PKC induced by externally added oxidants are inhibited by thiol agents, whereas the modification of PKC induced by calphostin C is not inhibited by thiol agents.

Ca²⁺-induced accessible hydrophobic sites are also present in other proteins such as calmodulin, calpains, S-100 protein, troponin C and other related Ca²⁺-regulated proteins [21,25,26]. It would be interesting to know whether calphostin C also could affect these proteins. Although calphostin C is often referred to as an inhibitor of PKC, to distinguish it from other inhibitors of PKC such as H-7, staurosporine, engermyrine, and sphingosine, which bind to and reversibly inhibit PKC, it should be treated as an irreversible inactivator of PKC. The irreversible inactivation of PKC may bring prolonged effects (hours) on the cells treated with calphostin C for a limited time. This information on irreversible inactivation may further help in experimental designs using calphostin C as a pharmacological tool and also in future therapeutic applications. Since this oxidative inactivation of PKC requires light, agents such as calphostin C may be useful in photodynamic therapy.

Acknowledgements. We thank Mr. Drake Jantzen for his excellent technical assistance. This work was supported by USPHS Grant CA 47142 from National Cancer Institute, Grant RT 388 from the Tobacco-Related Diseases Research Program, University of California, and a Research Grant from the Wright Foundation.

REFERENCES

- [1] Kikkawa, U., Kishimoto, A. and Nishizuka, Y. (1989) *Annu. Rev. Biochem.* 58, 31-44.
- [2] Sharkey, N., Leach, K. and Blumberg, P.M. (1984) *Proc. Natl. Acad. Sci. USA* 81, 607-610.
- [3] Housey, G.M., Johnson, M.D., Hsiao, W.L.W., O'Brian, C.A., Murphy, J.P., Kirschner, P. and Weinstein, I.B. (1988) *Cell* 52, 343-354.
- [4] Nield, J.E., Kuhn, L.J. and Vandenbark, G.R. (1983) *Proc. Natl. Acad. Sci. USA* 80, 36-40.
- [5] Melloni, E., Pontremoli, S., Michetti, M., Sacco, O. and Horecker, B.L. (1985) *Biochem. Biophys. Res. Commun.* 128, 331-338.
- [6] Gopalakrishna, R. and Anderson, W.B. (1989) *Proc. Natl. Acad. Sci. USA* 86, 6758-6762.
- [7] Gopalakrishna, R. and Anderson, W.B. (1987) *FEBS Lett.* 225, 233-237.
- [8] Gopalakrishna, R. and Anderson, W.B. (1991) *Arch. Biochem. Biophys.* 285, 382-387.
- [9] Delclos, K.B. and Blumberg, P.M. (1982) *Cancer Res.* 42, 1227-1232.
- [10] Kass, G.E.N., Duddy, S.K. and Orenius, S. (1989) *Biochem. J.* 260, 499-507.
- [11] Larsson, R. and Cerutti, P. (1989) *Cancer Res.* 49, 5627-5632.
- [12] Halliwell, B. (1989) *Br. J. Exp. Pathol.* 70, 737-757.
- [13] Tamaoki, T. (1991) *Methods Enzymol.* 201, 340-347.
- [14] Mahoney, C.W., Azzi, A. and Huang, K.P. (1990) *J. Biol. Chem.* 265, 5424-5428.
- [15] Hannun, Y.A. and Bell, R.M. (1987) *Science* 235, 670-674.
- [16] Kobayashi, E., Nakano, H., Morimoto, M. and Tamaoki, T. (1989) *Biochem. Biophys. Res. Commun.* 159, 548-553.
- [17] Brann, R.F., Miller, F.D., Murphree, R.L., Howbert, J.J., Hannun, W.F., Kobayashi, E., Takahashi, I., Tamaoki, T. and Nakano, H. (1991) *Biochem. Biophys. Res. Commun.* 176, 288-293.
- [18] Gopalakrishna, R., Barsky, S.H., Thomas, T.P. and Anderson, W.B. (1986) *J. Biol. Chem.* 261, 16438-16445.
- [19] Gopalakrishna, R. and Nagarajan, B. (1978) *Biochem. Med.* 22, 70-75.
- [20] Gopalakrishna, R., Chen, Z.H., Gundimeda, U., Wilson, J.C. and Anderson, W.B. (1992) *Anal. Biochem.* 206, 24-35.
- [21] Anderson, W.B. and Gopalakrishna, R. (1984) *Curr. Topics Cell. Reg.* 27, 455-469.
- [22] Puppo, A. and Halliwell, B. (1988) *Biochem. J.* 249, 185-190.
- [23] Youngman, R.J., Schieberle, P., Schnabl, H., Grosch, W. and Elstner, E.F. (1983) *Photobiochem. Photobiophys.* 6, 109-119.
- [24] Di Mascio, P., Kaiser, S., Devasagayam, T.P.A., Sundquist, A.R. and Sies, H. (1991) in: *Oxidative Damage and Repair* (Davies, K.J. ed.) pp. 311-314, Pergamon Press, Oxford, UK.
- [25] Gopalakrishna, R. and Anderson, W.B. (1982) *Biochem. Biophys. Res. Commun.* 104, 830-836.
- [26] Gopalakrishna, R. and Head, J.F. (1985) *FEBS Lett.* 186, 246-250.