Pergamon

*Biochemical Pharmacology*, Vol. 50, No. 2, pp. 235–241, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(95)00118-2

# INHIBITION OF DIACYLGLYCEROL KINASE BY THE ANTITUMOR AGENT CALPHOSTIN C

## EVIDENCE FOR SIMILARITY BETWEEN THE ACTIVE SITE OF DIACYLGLYCEROL KINASE AND THE REGULATORY SITE OF PROTEIN KINASE C

CLAIRE REDMAN,* JANE LEFEVRE† and MARNIE L. MACDONALD*‡

*The Foundation for Genetic Research, Phoenix, AZ 85007; and †Harrington Arthritis Research Center, Phoenix, AZ 85006, U.S.A.

(*Received* 13 *July* 1994; *accepted* 19 *January* 1995)

**Abstract**—Calphostin C is an anti-tumor agent that binds to the regulatory domain of protein kinase C and inhibits the binding of phorbol dibutyrate. Recent studies suggest that there may be structural similarities between protein kinase C (PKC) and diacylglycerol kinase (DGK). Both enzymes bind diacylglycerol and phosphatidylserine, and sequencing of the 80 kDa diacylglycerol kinase shows that it contains zinc finger-like sequences, similar to those occurring in PKC. Similarities in some enzymatic properties of PKC and DGK led us to examine whether regulatory-site inhibitors of PKC also might inhibit DGK. For these studies, the membrane-bound DGK was partially purified from porcine testis membranes. Calphostin C inhibited DGK with an $IC_{50}$ in the micromolar range. The inhibition of DGK by calphostin C was competitive with respect to diacylglycerol and was not affected by the presence or absence of phosphatidylserine. Other inhibitors of protein kinase C were without effect, with the exception of Adriamycin®, which inhibited at millimolar concentrations. Staurosporine, which binds to the catalytic domain of protein kinase C, did not inhibit DGK. The results suggest that there are functional similarities between the substrate binding site of DGK and the regulatory site of protein kinase C.

*Key words:* protein kinase C; diacylglycerol kinase; signal transduction

Diacylglycerol accumulates in cellular membranes in response to hormones that stimulate phospholipid breakdown. In addition to its central role as a precursor to phospholipids and triacylglycerol, diacylglycerol serves as a second messenger by binding to and activating PKC§ [1]. This signal is, in large part, attenuated by DGK, which phosphorylates diacylglycerol to form phosphatidic acid, thereby removing the source of PKC activation [2].

The regulation of PKC has received intense scrutiny. The molecule contains two domains: a catalytic domain at the carboxyl terminus, which binds ATP and protein substrate, and a regulatory domain at the amino terminus, which is structurally distinct from other protein kinases and has a binding region for diacylglycerol and $Ca^{2+}$/phospholipid [2]. Phorbol esters substitute for diacylglycerol at the regulatory site and induce a more prolonged activation that is thought to account for the tumor-promoting activity of the phorbol esters.

‡ Corresponding author: Dr. Marnie L. MacDonald, The Foundation for Genetic Research, 1120 West Watkins Road, Phoenix, AZ 85007. Tel. (602) 495-3807; FAX (602) 254-7340.

§ Abbreviations: PKC, protein kinase C; DGK, diacylglycerol kinase; DTT, dithiothreitol; ADR, Adriamycin®; PI, phosphatidylinositol; OG, octyl-β-D-glucopyranoside; and MOPS, 4-morpholinepropanesulfonic acid.

Because of the apparent role of PKC in tumorigenesis, much effort has been focused on identifying specific inhibitors of the enzyme. Several classes of compounds have emerged, including some that act primarily at the catalytic domain and others that act at the regulatory region of the protein. Staurosporine, the most potent inhibitor of protein kinase C, binds to the catalytic domain of PKC as well as other protein kinases and competes with ATP for binding [3]. ADR, polymyxin B and trifluoperazine inhibit PKC primarily by interfering with the activation of the enzyme by $Ca^{2+}$ and phospholipid [4–6]. A relatively new group of inhibitors includes calphostin C, which binds to the regulatory domain of PKC and inhibits the binding of phorbol dibutyrate [7] (see Fig. 1). Calphostin C, an anti-tumor agent, has been thought to be a highly specific inhibitor of PKC, since it has a negligible effect on other protein kinases, even at concentrations greater than 50 μM.

The enzymatic properties of DGK have been studied less than those of PKC, despite the central role of DGK in signal attenuation. DGK exists in multiple forms, both cytosolic and membrane-bound [8–10]. Antibodies to the major (80 kDa) cytosolic protein do not react with other isozymes [11]. The 80-kDa protein is a $Ca^{2+}$/phospholipid-dependent enzyme that contains EF-hand motifs [12–14]. This enzyme also contains zinc finger-like sequences very similar to those occurring in PKC.

We have studied both membrane-bound and

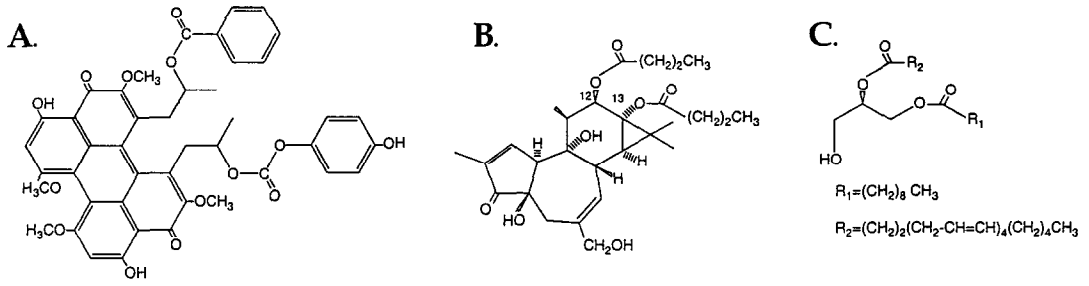


Fig. 1. Comparison of the structures of calphostin C (A), phorbol dibutyrate (B) and diacylglycerol (C).

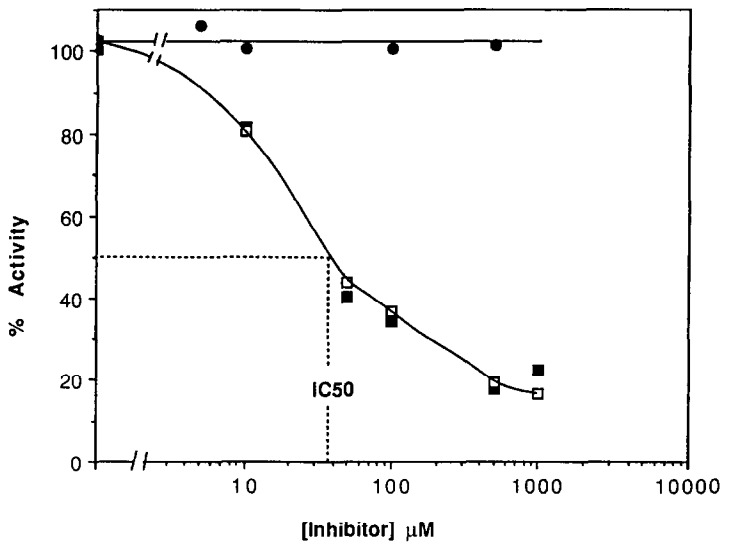


Fig. 2. Effects of calphostin C and staurosporine on DGK activity. DGK was preincubated with the reaction mixture containing 2 mol% diacylglycerol and increasing concentrations of staurosporine (●), calphostin C (■) or calphostin C in the presence of 2.9 mol% phosphatidylserine (□). Enzyme activity was measured as described in Materials and Methods. The activity due to the presence of endogenous diacylglycerols in the reaction mixture was determined by omitting diacylglycerol from the reaction and subtracting this value as background. The DGK activity measured in the absence of inhibitor was assigned the value of 100% (approximately 100 pmol/min/mg protein).

cytosolic forms of DGK that were purified from porcine testis. The membrane-bound form behaves as an arachidonoyl-specific integral membrane protein, previously found in 3T3 cells and baboon brain and testis, and has been shown to participate in the attenuation of diacylglycerol signal produced in response to platelet-derived growth factor [15].

Similarities in some enzymatic and structural properties of DGK and PKC led us to examine the effects of PKC inhibitors on DGK. The studies presented here were designed to investigate whether certain regulatory-site or catalytic-site inhibitors of PKC could also inhibit DGK.

## MATERIALS AND METHODS

*Materials.* [$\gamma$-$^{32}$P]ATP (3000 Ci/mmol) was obtained from Du Pont-New England Nuclear, Boston, MA, and 1-stearoyl-2-arachidonoyl-*sn* glycerol (DAG) was from Serdary Research Laboratories, Ontario, Canada. Bovine liver L-PI, egg L-phosphatidic acid and L-phosphatidylserine were purchased from Avanti Polar Lipids, Birmingham, AL. OG, trifluoperazine, and polymyxin B were obtained from Bochringer Mannheim, Indianapolis, IN; and tamoxifen, ADR, ATP, DTT, leupeptin, histone III-S and pepstatin A were from the Sigma Chemical Co., St. Louis, MO. Heparin Sepharose was purchased from Pharmacia-LKB Biotechnology, Piscataway, NJ; and hydroxylapatite was from Bio-Rad Laboratories, Richmond, CA. Calphostin C and staurosporine were obtained from the Kamiya Biomedical Co., Thousand Oaks, CA. Calphostin C was activated under fluorescent light as recommended

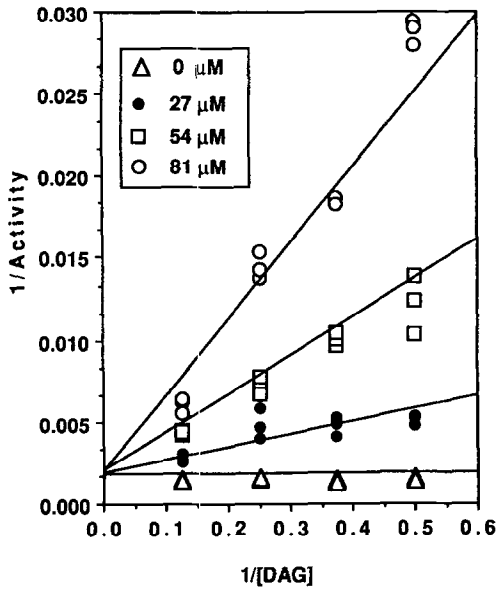


Fig. 3. Kinetics showing the competitive inhibition of membrane DGK with respect to diacylglycerol (DAG) by calphostin C. Membrane DGK was preincubated for 10 min at room temperature with several concentrations of calphostin C and increasing concentrations of DAG. The assay was initiated by the addition of [$\gamma$-$^{32}$P]ATP and terminated after 10 min. Calphostin C was present at 0, 27, 54 or 81 $\mu$M. Each point represents a single determination. Results shown are representative of three separate experiments. Units of activity are in picomoles per minute per milligram of protein.

by the manufacturer. Heparin was purchased from CalBiochem, San Diego, CA, and precoated cellulose plates were from EM Science, Cherry Hill, NJ. PKC was from Molecular Probes Inc., Eugene, OR.

*ADR–iron(III) complex.* This was prepared as described elsewhere [4]. Briefly, a 12 or 2 mM ADR solution in 10 mM formic acid was mixed, respectively, with an equal volume of a 4 or 0.67 mM ferric chloride solution. This mixture was titrated over a period of 2–3 hr to a pH of 7.5 with 10 mM sodium hydroxide.

*Purification of membrane DGK.* Unless otherwise indicated, all DGK purification steps were performed at 4°. Adult boar testes were decapsulated and homogenized in homogenization buffer consisting of 20 mM MOPS and 200 mM NaCl, pH 7.2, with 10 mM DTT, 4.5 mM ATP, 1 $\mu$g/mL leupeptin, 1 $\mu$g/mL pepstatin A and 50 $\mu$g/mL trypsin inhibitor. The homogenate was filtered through cheesecloth prior to centrifugation for 29 min at 3000 *g* in a Beckman T35 rotor. The supernatant was then centrifuged for 1 hr at 100,000 *g* in a Beckman T35 rotor. The resulting membrane pellet was dounced thoroughly in homogenization buffer and stored as 1-mL aliquots at −70°.

Membranes were extracted with an equal volume of extraction buffer [0.05 M Tris (pH = 7.4), 20% glycerol, 0.2 mM ATP, 2 mM DTT and 8% OG] for 30 min. Extracted membranes were centrifuged for 1 hr at 100,000 *g*, and the resulting supernatant was applied directly to a heparin Sepharose column equilibrated with 0.05 M Tris (pH = 7.4), 1% OG, 10% glycerol, 2 mM DTT and 0.02 mM ATP (wash buffer). Protein adhering to the heparin Sepharose was eluted directly onto hydroxylapatite with heparin (5 mg/mL) in wash buffer. A linear gradient of 0.15 M to 1.0 M potassium phosphate with 1% OG, 10% glycerol, 2 mM DTT and 0.1 mM ATP was used to elute DGK from the hydroxylapatite. Heparin was not detected in the hydroxylapatite eluate. Fractions were assayed for DGK activity, and the active fractions were pooled and adjusted to 30% glycerol prior to storage at −20° for no longer than 7 days. The resulting preparation exhibited specificity for arachidonoyl-diacylglycerol kinase, as shown previously [9, 10]. Elution profiles were comparable to previous results [9]. Complete details on the final purification and verification of the isoform are currently in preparation in a separate manuscript.

*Assay of DGK activity.* The activity of the DGK in the presence of the various PKC inhibitors was determined by measuring the incorporation of [$\gamma$-$^{32}$P]ATP into phosphatidic acid and determining the phosphorylation rate of diacylglycerol as described previously [10, 15]. Briefly, a 10-$\mu$L aliquot of DGK preparation was preincubated for 10 min at room temperature with the rest of the reaction mixture and 5 $\mu$L of inhibitor dissolved in $Me_2SO$, or $Me_2SO$ only as a control ($Me_2SO$ did not affect enzyme activity significantly). Following preincubation, the assay was initiated by the addition of [$\gamma$-$^{32}$P]ATP. Assays were terminated after 10 min at room temperature, and radioactivity incorporated into phosphatidic acid was determined following TLC of the reaction products on cellulose plates. Activity was linear over the 10-min assay time, and background activity resulting from endogenous diacylglycerol was insignificant (< 1% of total activity). To determine DGK activity in hydroxylapatite eluates as well as activity in the presence of ADR–iron(III) complexes, an abbreviated version of this same assay was used [8].

*Assay of PKC activity.* The activity of PKC in a mixed micellar assay system was determined by measuring the incorporation of [$\gamma$-$^{32}$P]ATP into calf thymus histone in the presence of Triton X-100 [16]. The 50-$\mu$L reaction contained 37 mM MOPS (pH 7.25), 12 mM $MgCl_2$, 1 mM $CaCl_2$, 40 $\mu$g histone (Sigma Type III-S) and mixed micelles. Mixed micelles were prepared by drying 10 $\mu$g phosphatidylserine and 0.8 $\mu$g diolein under nitrogen and redissolving thoroughly in 0.5% Triton X-100; a final concentration of 0.05% Triton X-100 was added. To assess background activity, $CaCl_2$ was replaced with 0.5 mM EGTA and 0.05% Triton X-100 only was added. In addition, every 5 $\mu$L of calphostin C was dissolved in $Me_2SO$; duplicate tubes with $Me_2SO$ only were used as a control. Finally, 10 ng of PKC was added to each reaction mixture, and the assay was initiated by the addition of [$\gamma$-$^{32}$P]ATP. Assays were terminated after 10 min at 30° by spotting 25 $\mu$L of the reaction mixture onto a Whatman P81 paper and washing the filter papers a total of three times

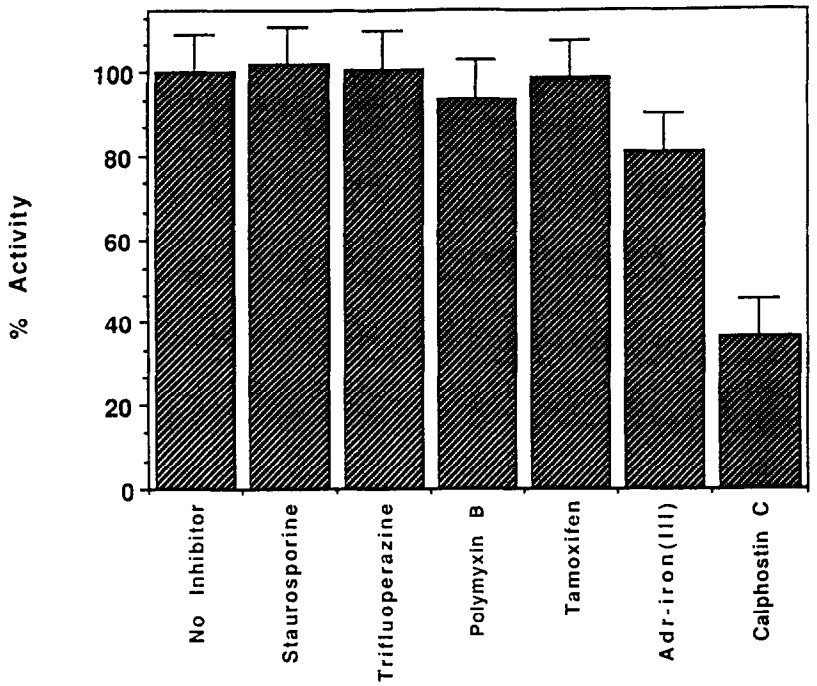


Fig. 4. Effects of various PKC inhibitors on DGK activity. Membrane DGK activity was measured in the presence of 2 mol% diacylgycerol, 2.9 mol% phosphatidylserine and 100 $\mu$M inhibitor. Activity in the presence of $Me_2SO$ only was assigned the value of 100% (approximately 100 pmol/min/mg protein). Each inhibitor was assayed in triplicate; values are means ± SD (N = 3).

in 250 mL of 75 mM $H_3PO_4$. The papers were dried, placed in scintillation vials, and counted.

## RESULTS

*Effect of calphostin C and staurosporine on DGK activity.* To determine if a regulatory-site inhibitor or a catalytic-site inhibitor of PKC might also inhibit DGK, the activity of DGK was examined in the presence of increasing concentrations of calphostin C or staurosporine (Fig. 2). Staurosporine, which inhibits protein kinases by competing with ATP for binding, was without effect on DGK at concentrations up to 1 mM. In contrast, calphostin C inhibited DGK activity with an $IC_{50}$ of approximately 40 $\mu$M. The inhibition of DGK by calphostin C was not affected by the presence or absence of phosphatidylserine, as shown.

*Kinetics of the inhibition of DGK by calphostin C.* To examine the mechanism of inhibition of DGK by calphostin C, DGK activity was measured at four concentrations of diacylglycerol (from 2 to 8 mol% of micelles) in the presence of either 0, 27, 54 or 81 $\mu$M calphostin C. Figure 3 shows results that were representative of three separate experiments. The results demonstrate that the inhibition of DGK by calphostin C was competitive with respect to diacylglycerol, as would be expected if calphostin C binds to the active (diacylglycerol binding) site of the enzyme.

*Comparison of effects of various PKC inhibitors on DGK activity.* Other regulatory-site inhibitors of PKC were tested for potential inhibition of DGK. These included ADR, polymyxin B, tamoxifen and trifluoperazine. ADR, in the presence of heavy metals, has been shown to inhibit PKC by competing with diacylglycerol. In contrast, the other compounds that were tested have been shown to inhibit PKC by interfering with the binding of phospholipid to PKC [4–6, 17]. Figure 4 shows that, of all the PKC inhibitors tested, only calphostin C gave a significant (70%) inhibition of DGK. However, ADR complexed with iron appeared to inhibit DGK moderately (about 20%), while the other compounds were completely without effect.

*Effects of ADR, ADR with $FeCl_3$, and ADR–iron(III) complexes on DGK activity.* To further examine the potential effect of ADR on DGK activity, ADR was tested at concentrations up to 1 mM. Figure 5A shows that DGK activity was progressively inhibited as the concentration of ADR alone was increased. At a concentration of 1 mM, ADR inhibited DGK activity by approximately 50%. To further delineate the role of heavy metal inhibition of enzyme activity, DGK activity was measured in the presence of ADR alone, $FeCl_3$ alone, ADR with $FeCl_3$, or ADR complexed with iron (3:1). As shown in Fig. 5B, $FeCl_3$ alone had no effect on enzyme activity. In contrast, ADR with or without iron caused up to a 25% inhibition of enzyme activity at concentrations of 100 $\mu$M inhibitor, and up to 70% inhibition at concentrations of 1 mM inhibitor.

*Effects of calphostin C on PKC activity in a mixed micellar assay.* Direct comparison of the $IC_{50}$ for

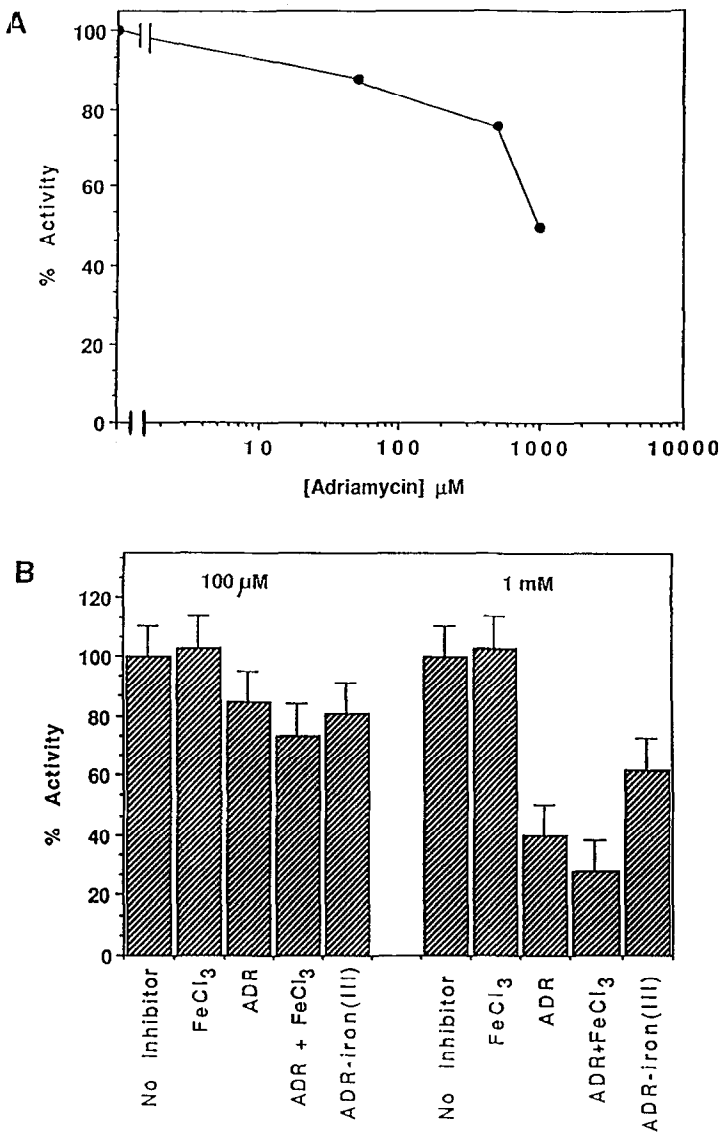


Fig. 5. Inhibitory effect of ADR and ADR–iron(III) complex on DGK activity. (A) Activity was measured in the presence of 2 mol% diacylglycerol, 2.9 mol% phosphatidylserine, and increasing concentrations of ADR, as shown. Each point is the mean of three determinations. (B) DGK activity (100% is approximately 100 pmol/min/mg) was determined as above with either $FeCl_3$ (38 or 186 μM), or ADR–iron(III) complex (3:1). Values are means ± SD (N = 3).

PKC and DGK required evaluation of the inhibition of PKC by calphostin C under mixed micellar conditions. Previous published results on the inhibition of PKC by calphostin had been based on a lipid disbursion that contained no detergent. Those results had shown an $IC_{50}$ for calphostin C in the nanomolar range. To evaluate the kinetics of the inhibition of PKC by calphostin C, we utilized a mixed micellar assay containing Triton X-100 as detergent.

Figure 6 shows that calphostin C inhibited PKC in the mixed micellar assay to a level of 40% of maximal activity. The lack of 100% inhibition might suggest that other histone phosphorylating enzymes were present in the preparation, despite correction of background by subtracting activity in the absence of $Ca^{2+}$/phospholipid; however, since the PKC utilized is nearly homogeneous, it is more likely that calphostin C only partially inhibits PKC in mixed micelles. Analysis of the $IC_{50}$, assuming 100% inhibition, showed a maximum $IC_{50}$ of 90 μM. If 50% of the fully inhibited enzyme was used as an indication of $IC_{50}$, the value would be equivalent to 50 μM. Therefore, the $IC_{50}$ in the mixed micellar assay ranged from 50 to 90 μM, a value that is 1000 times the published result with a mixed non-micellar assay. Moreover, this $IC_{50}$ result of 50–90 μM was similar to that obtained with DGK (Fig. 2). These

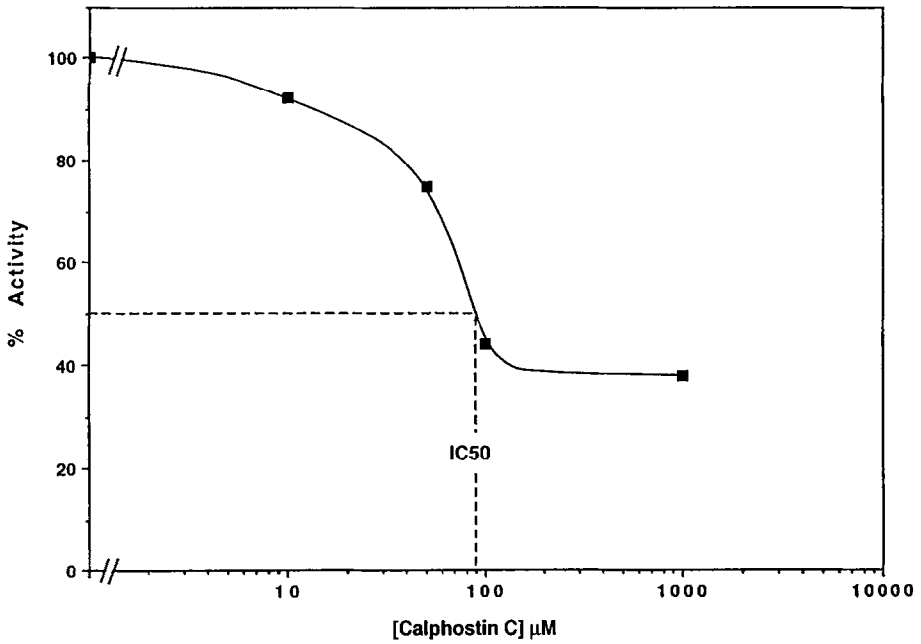


Fig. 6. Inhibition of protein kinase C by calphostin C in a mixed micellar assay. PKC was assayed with increasing concentrations of calphostin C in the presence of diacylglycerol/phosphatidylserine/detergent mixed micelles. The PKC activity in the absence of inhibitor was assigned the value of 100% (approximately 0.5 μmol/min/mg protein).

Table 1. Comparison of the structural and functional properties of protein kinase C (PKC) and diacylglycerol kinase (DGK)

| | PKC | DGK |
|---|---|---|
| Substrates | Protein/ATP | Diacylglycerol/ATP |
| DAG binding | + | + |
| Phospholipid activation | + | + |
| Phorbol diester binding | + | − |
| Calphostin C inhibition | + | + |
| | ($IC_{50}$ = 50–90 μM)* | ($IC_{50}$ = 40 μM)* |
| | ($IC_{50}$ = 50 nM) (7) | Not determined |
| Adriamycin inhibition | + | + |
| | ($IC_{50}$ = 13 μM) | ($IC_{50} \geq$ 1 mM) |
| Staurosporine inhibition | + | − |

* Under mixed micellar conditions.

results suggest that under mixed micellar conditions, the $IC_{50}$ of calphostin C for DGK is similar to that for PKC.

## DISCUSSION

The results of these studies, and of studies of the structural properties of PKC and DGK, are summarized in Table 1. The results demonstrate that calphostin C and ADR, compounds that have been shown previously to inhibit protein kinase C by binding to the regulatory site of that enzyme, also inhibit DGK.

The $IC_{50}$ of calphostin C has been shown to be 50 nM with respect to protein kinase C activity in assays lacking detergent. Our studies showed, however, that the $IC_{50}$ was approximately 1000-fold higher under mixed micellar conditions (Fig. 6). Therefore, the $IC_{50}$ of calphostin C may well be similar for both DGK (40 μM) and PKC (50–90 μM).

The difference in apparent $IC_{50}$ for PKC under mixed micellar versus non-detergent conditions might be explained by surface dilution of calphostin C in the mixed micelles. Detailed analysis would be required to determine how calphostin C partitions in a mixed micelle system and to assess the effective concentration of calphostin C at the micellar surface. For the purposes of this study, our results simply

indicate that the inhibition of DGK by calphostin C may be of significance in studies of the physiological effects of this inhibitor. In particular, previous studies of the physiological role of PKC may have been confounded by inhibition of DGK, especially with concentrations of calphostin C in excess of 1 $\mu$M.

The effects of calphostin C on DGK appear to be competitive with respect to diacylglycerol and are unaffected by the presence or absence of phosphatidylserine activator. These findings are consistent with a mechanism wherein calphostin C binds to the active (diacylglycerol-binding) site of the enzyme. The results suggest that there are similarities in functional properties of the diacylglycerol-binding (active) site of DGK and the diacylglycerol-binding (regulatory) site of PKC.

In contrast, staurosporine, a compound that broadly inhibits protein kinases (including PKC) by acting at the ATP-binding site, was without effect on DGK, which indicates functional disparity between the active sites of PKC and DGK, as would be anticipated from the marked difference in substrate preferences.

Although these results suggest some functional similarities between the diacylglycerol binding sites of the two enzymes, there are significant differences. PKC avidly binds phorbol diesters in addition to diacylglycerols. The substitution of diacylglycerols for phorbol esters requires the presence of the 3′-hydroxyl group, and medium-chain diacylglycerols are most effective, both *in vitro* and *in vivo*. In contrast, different DGK isozymes exhibit greater specificity for diacylglycerol binding. DGK isolated from membrane sources shows marked specificity for diacylglycerol chain length and polyunsaturation [10]. Most notably, neither cytosolic nor membrane forms of DGK have been found to bind phorbol diesters [12, 18]. Direct comparison of the structures of calphostin C, phorbol dibutyrate and diacylglycerol (Fig. 1) reveals similar free hydroxyl moieties that may be critical for the binding of all three compounds. However, while phorbol dibutyrate and calphostin C share a ring structure and are both avidly bound by PKC, this structure is lacking in diacylglycerol itself. Therefore, it seems likely that the high degree of specificity exhibited by DGK for its substrate precludes the binding of phorbol dibutyrate and may also account for the less potent inhibition of DGK by calphostin C. Further studies of the functional properties of DGK may aid in the development of specific inhibitors.

*Acknowledgements*—These studies were supported by the Arizona Disease Control Research Commission and the National Institutes of Health.

## REFERENCES

1. Parker PJ, Coussens L, Totty N, Rhee L, Young S, Chen E, Stabel S, Waterfield MD and Ullrich A, The complete primary structure of protein kinase C—the major phorbol ester receptor. *Science* **233**: 853–859, 1986.
2. Bishop WE, Ganong BR and Bell RM, Attenuation of *sn*-1,2-diacylglycerol second messengers by diacylglycerol kinase. Inhibition by diacylglycerol analogs *in vitro* and in human platelets. *J Biol Chem* **261**: 6993–7000, 1986.
3. Tamaoki T and Nakano F, Potent and specific inhibitors of protein kinase C of microbial origin. *Biotechnology* **8**: 732–735, 1990.
4. Hannun YA, Foglesong RJ and Bell RM, The adriamycin–iron (III) complex is a potent inhibitor of protein kinase C. *J Biol Chem* **264**: 9960–9966, 1989.
5. Mazzei GJ, Katoh N and Kuo JF, Polymyxin B is a more selective inhibitor for phospholipid-sensitive $Ca^{2+}$-dependent protein kinase than for calmodulin-sensitive $Ca^{2+}$-dependent protein kinase. *Biochem Biophys Res Commun* **109**: 1129–1133, 1982.
6. Wise BC and Kuo JF, Modes of inhibition by acylcarnitines, adriamycin and trifluoperazine of cardiac phospholipid-sensitive calcium-dependent protein kinase. *Biochem Pharmacol* **32**: 1259–1265, 1983.
7. Kobayashi E, Nakano H, Morimoto M and Tamaoki T, Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. *Biochem Biophys Res Commun* **159**: 548–553, 1989.
8. Stathopoulos VM, Coco-Maroney A, Wei C-W, Goth M, Zaricznyj C and Macara IG, Identification of two cytosolic diacylglycerol kinase isoforms in rat brain, and in NIH-3T3 and *ras*-transformed fibroblasts. *Biochem J* **272**: 569–575, 1990.
9. Lemaitre RN, King WC, MacDonald ML and Glomset JA, Distribution of distinct arachidonoyl-specific and non-specific isoenzymes of diacylglycerol kinase in baboon (*Papio cynocephalus*) tissues. *Biochem J* **266**: 291–299, 1990.
10. MacDonald ML, Mack KF, Williams BW, King WC and Glomset JA, A membrane-bound diacylglycerol kinase that selectively phosphorylates arachidonoyl-diacylglycerol. Distinction from cytosolic diacylglycerol kinase and comparison with the membrane-bound enzyme from *Escherichia coli*. *J Biol Chem* **263**: 1584–1592, 1988.
11. Yamada K and Kanoh H, Occurrence of immunoreactive 80 kDa and non-immunoreactive diacylglycerol kinases in different pig tissues. *Biochem J* **255**: 601–608, 1988.
12. Sakane F, Yamada K, Kanoh H, Yokoyama C and Tanabe T, Porcine diacylglycerol kinase sequence has zinc finger and E-F hand motifs. *Nature* **344**: 345–348, 1990.
13. Sakane F, Yamada K, Imai S and Kanoh H, Porcine 80-kDa diacylglycerol kinase is a calcium-binding and calcium/phospholipid-dependent enzyme and undergoes calcium-dependent translocation. *J Biol Chem* **266**: 7096–7100, 1991.
14. Schaap D, de Widt J, van der Wal J, Vandekerckhove J, van Damme J, Gussow D, Ploegh HL, van Blitterswijk WJ and van der Bend RL, Purification, cDNA-cloning and expression of human diacylglycerol kinase. *FEBS Lett* **275**: 151–158, 1990.
15. MacDonald ML, Mack KF, Richardson CN and Glomset JA, Regulation of diacylglycerol kinase reaction in Swiss 3T3 cells. *J Biol Chem* **263**: 1575–1583, 1988.
16. Kikkawa U, Takai Y, Minakuchi R, Inohara S and Nishizuka Y, Calcium-activated, phospholipid-dependent protein kinase from rat brain. Subcellular distribution, purification, and properties. *J Biol Chem* **257**: 13341–13348, 1982.
17. O'Brien CA, Liskamp RM, Solomon DH and Weinstein IB, Inhibition of protein kinase C by tamoxifen. *Cancer Res* **45**: 2462–2465, 1985.
18. Besterman JM, Pollenz RS, Booker EL Jr and Cuatrecasas P, Diacylglycerol-induced translocation of diacylglycerol kinase: Use of affinity-purified enzyme in a reconstitution system. *Proc Natl Acad Sci USA* **83**: 9378–9382, 1986.