Biochemical Pharmacology, Vol. 59, pp. 763–772, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(99)00395-0

Selectivity of the Diacylglycerol Kinase Inhibitor 3-{2-(4-[bis-(4-Fluorophenyl)methylene]-1-piperidinyl)ethyl}-2,3-dihydro-2-thioxo-4(1*H*)quinazolinone (R59949) among Diacylglycerol Kinase Subtypes

Ying Jiang, Fumio Sakane,† Hideo Kanoh† and James P. Walsh*‡*

*Departments of Medicine and Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN, U.S.A.; and the †Department of Biochemistry, Sapporo Medical University School of Medicine, Sapporo, Japan

ABSTRACT. Diacylglycerol kinases (DGKs) attenuate diacylglycerol-induced protein kinase C activation during stimulated phosphatidylinositol turnover. This reaction also initiates phosphatidylinositol resynthesis. Two agents, 3-{2-(4-[bis-(4-fluorophenyl)methylene]-1-piperidinyl)ethyl}-2,3-dihydro-2-thioxo-4(1*H*)quinazolinone (R59949) and 6-{2-{4-[(4-fluorophenyl)phenylmethylene]-1-piperidinyl}ethyl}-7-methyl-5*H*-thiazolo(3,2-*a*)pyrimidin-5-one (R59022), inhibit diacylglycerol phosphorylation in several systems. To examine the mechanism of this effect, we developed a mixed micelle method suitable for *in vitro* study of DGK inhibition. Animal cells express multiple DGK isoforms. In a survey of DGK isotypes, these agents selectively inhibited Ca^{2+}-activated DGKs. R59949 was the more selective of the two. To map the site of interaction with the enzyme, a series of DGKα deletion mutants were prepared and examined. Deletion of the Ca^{2+}-binding EF hand motif, which is shared by Ca^{2+}-activated DGKs, had no effect on inhibition. Consistent with this observation, inhibition kinetics were noncompetitive with Ca^{2+}. A construct expressing only the catalytic domain was also inhibited by R59949. Studies of substrate kinetics demonstrated that MgATP potentiated R59949 inhibition, indicating synergy of inhibitor and MgATP binding. These results indicate that R59949 inhibits DGKα by binding to its catalytic domain. BIOCHEM PHARMACOL **59**;7:763–772, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. diacylglycerol; phosphotransferases; phosphatidic acid; calcium

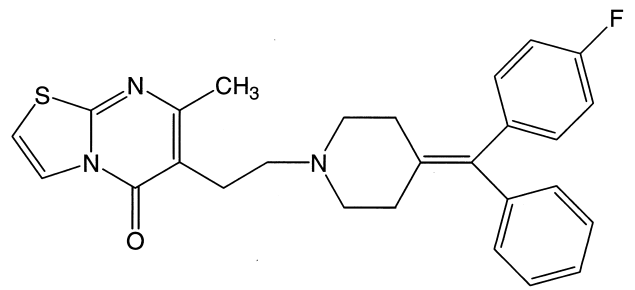
DGKs§ phosphorylate DAG and thereby attenuate DAG-mediated PKC activation [1–3]. DGKs also modulate levels of PA, another lipid mediator [3]. Activation of DGKs through pathways not involving phosphoinositide turnover or PKC activation has also been described [4]. DGKs are a large family of isoenzymes [2, 3]. All of them share a conserved catalytic domain. They also contain C1 PKC homology domains, which may target DGKs to surfaces via specific interactions with membrane lipids [2, 5]. Some DGKs contain Ca^{2+}-binding EF hand motifs, which presumably mediate Ca^{2+} activation of these isoforms [1, 6]. Other motifs found in DGKs include ankyrin repeats and pleckstrin homology domains [2, 3]. Understanding the roles of DGKs is complicated by the fact that individual cells express multiple isoforms. T-lymphocytes, for example, express at least four DGKs [7]. Moreover, not all cellular DAG is accessible to DGKs [8]. This suggests that, as with PKCs, DGKs are targeted to specific intracellular addresses where they phosphorylate specific DAG pools. In this regard, several groups have reported evidence for stimulus-induced association of DGKs with specific particulate fractions [4, 9–11].

Two agents, 3-{2-(4-[bis-(4-fluorophenyl)methylene]-1-piperidinyl)ethyl}-2,3-dihydro-2-thioxo-4(1*H*)quinazolinone (R59949) and 6-{2-{4-[(4-fluorophenyl)phenylmethylene]-1-piperidinyl}ethyl}-7-methyl-5*H*-thiazolo-(3,2-*a*)pyrimidin-5-one (R59022) (Fig. 1), inhibit DAG phosphorylation by erythrocyte ghosts and platelet membranes [12, 13]. These compounds also inhibit *in vivo* phosphorylation of exogenous short-chain DAG analogs by intact platelets [12–14] and guinea pig taenia coli [15]. R59022 potentiates fMetLeuPhe-induced superoxide production by neutrophils [16, 17], thrombin-induced platelet aggregation and secretion [14], IgE-induced histamine release from mast cells [18], and cholecystokinin-induced pancreatic enzyme secretion [19]. These effects have been attributed to a prolongation of DAG-induced PKC activa-

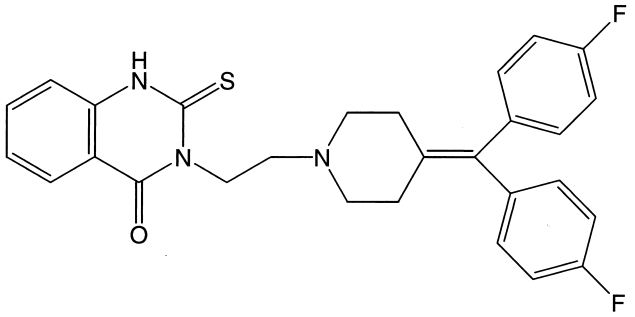
‡ Corresponding author: Dr. James P. Walsh, Section of Endocrinology and Metabolism (111-E), Roudebush VA Medical Center, 1481 West Tenth Street, Indianapolis, IN 46202. Tel. (317) 554-0000, Ext. 3073; FAX (317) 554-0004; E-mail: jpwalsh@iupui.edu

§ *Abbreviations*: BHT, 2,6-di-*tert*-butyl-4-methylphenol; DAG, *sn*-1,2-diacylglycerol; DGK, diacylglycerol kinase; DTPA, diethylenetriaminepentaacetic acid; DTT, dithiothreitol; OTAC, octadecyltrimethylammonium chloride; PA, phosphatidic acid; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; and PS, phosphatidylserine.

Received 10 June 1999; accepted 24 August 1999.



R59022



R59949

FIG. 1. Structures of R59022 (6-{2-{4-[(4-fluorophenyl)phenylmethylene]-1-piperidinyl}ethyl}-7-methyl-5*H*-thiazolo(3,2-*a*)pyrimidin-5-one) and R59949 (3-{2-(4-[bis-(4-fluorophenyl)-methylene]-1-piperidinyl)ethyl}-2,3-dihydro-2-thioxo-4(1*H*)-quinazolinone).

tion. R59949 and R59022 inhibit interleukin-2-induced lymphocyte proliferation [4], carbachol-induced smooth muscle contraction [15], and platelet-derived growth factor-induced vascular smooth muscle proliferation [20]. These inhibitory effects may reflect decreased phosphatidate-mediated signaling. Both prolonged PKC activation and decreased PA signaling would be consistent with inhibition of a DGK [3].

Some, but not all, DGK preparations are inhibited by R59949 or R59022 [21–23]. These studies employed various assay methods, rendering comparisons difficult. Another confounding factor is the use of anionic lipids as activators in many DGK assays. Intramicellar complexation with cationic moieties on R59949 and R59022 may reverse activation by anionic lipids. Reversal of lipid activation may be difficult to distinguish from direct inhibition. We now have developed a mixed micellar assay that overcomes these problems and have demonstrated that R59949 and R59022 inhibit DGKs in direct proportion to their *in vivo* potency. Our results confirm that DGKs are direct targets of R59949 and R59022. Surprisingly, this inhibition was selective for calcium-activated DGK isoforms. However, inhibition did not require EF hand motifs unique to Ca^{2+}-activated DGKs.

MATERIALS AND METHODS

Materials

[γ-^{32}P]ATP was from DuPont New England Nuclear. Octyl-β-d-glucopyranoside (octylglucoside), sodium deoxycholate, PMSF, Triton X-100, Triton X-114, leupeptin, aprotinin, DTPA, *N*-hexadecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate (C16 sulfobetaine, C16SB), EDTA, EGTA, dihexadecyl phosphate, and sorbitan trioleate were from the Sigma Chemical Co. BHT was from the Aldrich Chemical Co. *sn*-1-Palmitoyl-2-oleoyl phosphatidylserine (PS) was from Avanti Polar Lipids. *sn*-1-Palmitoyl-2-oleoylglycerol (16:0,18:1-DAG) was prepared by digestion of the corresponding phosphatidylcholine (Avanti) with *Bacillus cereus* phospholipase C [24]. DE52 cellulose was from Whatman. SP-Sepharose Fast Flow and Q-Sepharose Fast Flow were from Pharmacia. The DGK inhibitors R59022 and R59949 (Diacylglycerol Kinase Inhibitor I and Diacylglycerol Kinase Inhibitor II) were from Calbiochem.

Purification of DGKs

To facilitate comparison of enzymologic properties, DGKs were purified from a variety of sources. Marked differences in the effects of Ca^{2+} and phospholipids, together with the fact that DGKα (the thymus A DGK isoform) is highly expressed only in brain and thymocytes, indicate that these activities reflect distinct DGK isoenzymes [25–27]. Testis cytosol DGK, NIH 3T3 cell DGKs A and B, and arachidonoyl DGK from testis membranes were prepared as described previously [22, 25]. Thymus DGKs were prepared by a modification of previously described methods [21, 25]. Porcine thymi (Pel-Freez) were homogenized in 25 mM Tris–HCl, pH 7.4, 250 mM sucrose, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 1 μg/mL of leupeptin, 1 μg/mL of aprotinin, 1 mM benzamidine, 1 mM PMSF, and 0.02% Triton X-100, and centrifuged at 3000 *g* for 15 min. The supernatant was passed through cheesecloth and glass wool and then was centrifuged at 100,000 *g* for 75 min. This supernatant (525 mL) was loaded onto a 290-mL DE52 cellulose column that had been equilibrated previously with the extraction buffer. The column was washed with 400 mL of the same buffer and then eluted with 300 mL of buffer containing 100 mM NaCl. Peak fractions were pooled as Fraction A. The column was eluted further with 300 mL of buffer containing 200 mM NaCl, and peak fractions were pooled as Fraction B. Pooled Fraction A (5 mL) was loaded onto a 10-mL SP-Sepharose Fast Flow column equilibrated with buffer containing 100 mM NaCl. After washing with 12 mL of the equilibration buffer, the column was eluted by a 26-mL gradient of 100–400 mM NaCl in the same buffer. Fractions were assayed for DGK activity, and active fractions, which eluted at 150 mM NaCl, were pooled and frozen as thymus A. Fraction B (5 mL) was diluted to 100 mM NaCl and loaded onto a 25-mL SP-Sepharose Fast Flow column equilibrated with buffer containing 100 mM NaCl. The column was washed with 75 mL of equilibration

buffer and eluted with a 100-mL gradient of 100–800 mM NaCl in the same buffer. Active fractions, which eluted at 200 mM NaCl, were pooled as thymus B. To prepare salivary DGK, porcine submaxillary glands (Pel-Freez) were homogenized and centrifuged as described above. The cytosolic supernatant (150 mL) was loaded onto a 150-mL DE52 cellulose column equilibrated with extraction buffer. After washing with 50 mL of the equilibration buffer, the column was eluted by a 100-mL gradient of 50–200 mM NaCl. Active fractions, which eluted at 90 mM NaCl, were pooled and frozen at −70°.

DGK Assays

Several mixed micellar assays were employed to evaluate kinetic properties of DGKs. All of these methods gave activities that were linear with the times and protein concentrations employed. Calcium activation of DGKs was assessed with octylglucoside/PS/DAG mixed micelles. Calcium stimulation in this system was 3- to 4-fold greater than in the Triton/C16SB assay described below. Calcium activation of DGKs could not be detected with the Triton/OTAC or deoxycholate assays. The assay included 50 mM triethanolamine–HCl, pH 7.4, 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 45 mM octylglucoside, 2.5 mM (10 mol%) PS, 0.5 mM (2 mol%) 16:0,18:1-DAG, 0.1 mM [γ-^{32}P]ATP, and enzyme. Total concentrations of Ca^{2+} and Mg^{2+} added to assays were calculated as described from published stability constants to give 1.0 mM free Mg^{2+} and 0–50 μM free Ca^{2+} in the presence of 1 mM EDTA and 0.1 mM ATP [28]. Stock solutions of 4× octylglucoside/PS, 10x [γ-^{32}P]ATP, and 4× aqueous buffer were as described previously [24]. Mole fractions of PS and DAG were calculated as described [25]. The reaction was initiated by the addition of [γ-^{32}P]ATP and allowed to proceed for 10 min at 25°. Then it was terminated by the addition of 3 mL $CHCl_3$:ethanol (2:1, v/v) containing 1 mg dihexadecylphosphate and 1 mg sorbitan trioleate [25]. The organic phase was washed three times with 2 mL of 1.0% $HClO_4$, 0.1% H_3PO_4 in H_2O:ethanol (7:1, v/v) [25]. Then incorporation of ^{32}P into phosphatidic acid was determined by Cerenkov counting of 1.2 mL of the organic phase. DGK inhibition by R59022 and R59949 was measured in a new assay that contained Triton and the zwitterionic detergent C16SB. This assay included 6 mM Triton X-100, 6 mM Triton X-114, 3 mM C16SB, 0.3 mM (2 mol%) 16:0,18:1-DAG, 50 mM triethanolamine–HCl, pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 0.1 mM [γ-^{32}P]ATP, and inhibitor as indicated. A 4× Triton/C16SB stock solution was prepared by dissolving C16SB in a 1:1 mixture of Triton X-100/Triton X-114. The Triton X-100/X-114 mixture was employed because its surface behavior is more favorable for DGKs than Triton X-100 alone [22]. The Triton X-100/X-114 stock solution was prepared and purified as described previously [25]. Ethanolic solutions of R59022 or R59949 were evaporated in the reaction tubes and dispersed into micelles by brief sonication in a water bath sonifier. Sonication was not required to solubilize the inhibitors, but did accelerate their dispersal. Then enzyme was added, and the reaction was initiated by the addition of [γ-^{32}P]ATP. After 10 min at 25°, the [^{32}P]phosphatidic acid was extracted and counted as described above. Effects of DGK inhibitors were also investigated in an assay that contained Triton and OTAC instead of C16SB. This assay was identical to the Triton/C16SB method, except that the concentrations of Triton X-100 and X-114 were reduced to 1.6 mM each, the 16:0,18:1-DAG was reduced to 0.08 mM (2 mol%), and the C16SB was replaced with 0.8 mM OTAC (20 mol%). To assay DGK activities of chromatographic fractions during purifications, a deoxycholate assay was employed. This assay included 1 mM deoxycholate, 20 μM 16:0,18:1-DAG, 50 mM triethanolamine–HCl, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, and 0.5 mM [γ-^{32}P]ATP.

Expression of DGKs in COS-1 Cells

Constructs for expression of full-length DGKα (pSRE-DGKα), and two truncation mutants, DGKα Δ196 (pSRE-DGKΔ196) and DGKα Δ332 (pSRE-DGKΔ332), have been described previously [6, 29]. cDNA for DGKζ (pSRE-DGK IV) was from Dr. K. Goto [30]. COS-1 cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 10% fetal bovine serum (Intergen), 100 U/mL of penicillin, and 100 μg/mL of streptomycin sulfate. COS-1 transfection was carried out using the calcium phosphate method [31]. After 48 hr, the cells were harvested and lysed by sonication in 0.5 mL per 100-mm dish of 20 mM Tris, pH 7.4, 0.25 M sucrose, 100 mM NaCl, 5 mM EGTA, 1 mM PMSF, 10 μg/mL of leupeptin, 10 μg/mL of aprotinin, 1 mM $MgCl_2$, 50 μM ATP, and 1 mM DTT. Undisrupted cells were removed by brief centrifugation, and the resulting cell lysates were centrifuged at 100,000 *g* for 20 min. The cytosolic supernatants were collected, aliquoted, and frozen at −70° until assayed.

Expression of DGKs in Yeast

To express DGKα constructs in *Saccharomyces cerevisiae*, *Eco*RI fragments containing the desired sequences were subcloned into pYCDE2 and introduced into *S. cerevisiae* strain WY294 (*MAT*α *GCN2 ino1 ura3–52 leu2–3 leu2–112 trp1 HIS4-lacZ*) [32]. Transformed cells were grown in 2 L of synthetic dextrose minimal medium supplemented with 20 mg/L of uracil, 30 mg/L of leucine, 30 mg/L of isoleucine, and 150 mg/L of valine [33]. Cells were collected by centrifugation and disrupted with glass beads in 25 mM Tris–HCl, pH 7.4, 50 mM NaCl, 0.25 M sucrose, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM DTT, 50 μM ATP, 0.02% Triton X-100, 2 μg/mL of aprotinin, 2 μg/mL of leupeptin, 3 μg/mL of pepstatin, 1 mM benzamidine, and 1 mM PMSF. Debris and unbroken cells were removed by

centrifugation at 5000 *g* for 15 min, and the supernatant was applied to a 10-mL DEAE–cellulose column equilibrated with lysis buffer. The column was washed with 20 mL of this same buffer and eluted with a 20-mL linear gradient of 50–200 mM NaCl at a flow rate of 0.5 mL/min. The Δ196 and Δ332 DGKs were unstable to further purification. Full-length DGKα was purified further to apparent homogeneity by the following procedure. DEAE fractions with peak activity were pooled, adjusted to 100 mM NaCl, and loaded onto a 10-mL SP-Sepharose Fast Flow column equilibrated in lysis buffer containing 100 mM NaCl. The column was washed with an additional 20 mL of this buffer and then eluted with a 40-mL linear gradient of 100–400 mM NaCl at a flow rate of 0.5 mL/min. Active fractions were pooled, adjusted to 100 mM NaCl, and applied to a 10-mL Q-Sepharose Fast Flow column equilibrated with buffer containing 100 mM NaCl. The column was washed with 10 mL of this buffer and eluted with a 20-mL gradient of 100–600 mM NaCl at a flow rate of 0.5 mL/min. Active fractions were pooled, rapidly frozen in dry ice/ethanol, and stored at −70° until assayed. All purification steps were performed at 4°.

Data Handling

Activities reported are averages of duplicate assays, which in all cases agreed within 10%. All results shown are representative of two or more separate experiments performed with completely independent enzyme preparations. Kinetic constants for DGKα inhibition by R59949 were obtained by fitting activities to the appropriate rate equation using a nonlinear least squares (Marquardt–Levenberg) algorithm supplied with the SigmaPlot software package.

RESULTS

Selectivity of R59949 for Ca^{2+}-Activated DGKs

R59022 and R59949 (Fig. 1) were initially described as inhibitors of DAG phosphorylation in intact cells [12, 13]. However, *in vitro* inhibition of DGK activities has not been observed uniformly. One potential difficulty with such measurements is complexation of cationic moieties on these compounds with anionic lipid cofactors employed in DGK assays. We have also noted these agents to be poorly soluble in octylglucoside and deoxycholate, which are commonly used to disperse the DAG substrate. Triton is a more potent solubilizer than octylglucoside or deoxycholate, but some DGKs are inhibited by this detergent, even at low concentrations [23]. We recently noted that addition of 20 mol% of the cationic detergent OTAC to Triton micelles overcame Triton inhibition of several DGKs and yielded activities comparable to those in a standard octylglucoside/PS assay [22]. However, this assay interfered with measurements of Ca^{2+} activation (data not shown). A survey of other amphiphiles demonstrated that 20 mol% of the zwitterionic detergent C16SB in Triton gave DGKα activities similar to other assays. Calcium

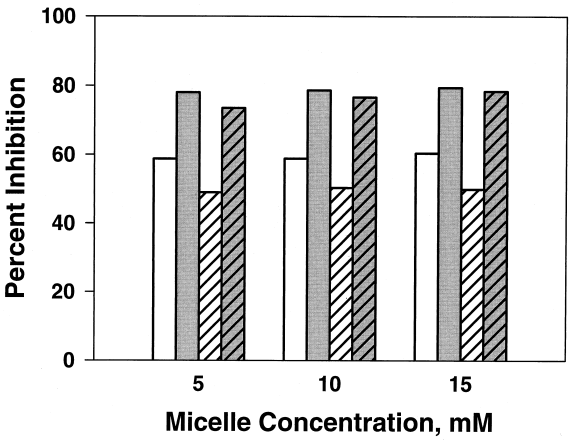


FIG. 2. DGKα inhibition by R59022 and R59949 at different micelle concentrations. Effects of micelle concentration on inhibition of DGKα from thymus by R59022 and R59949 were examined. The sum of the concentrations of Triton, C16SB, DAG, and inhibitor was 5, 10, or 15 mM as shown. Proportions of micelle components were kept constant throughout. C16SB was 20 mol% and DAG 2 mol%. R59022 was 2 or 8 mol% and R59949 was 0.4 or 2 mol%. These assays were done using DGKα prepared from thymus. The specific activity of this preparation in the Triton/C16SB assay was 17.6 nmol/min/mg protein. Duplicate assays were performed in each of two separate experiments. Activities are expressed as percentages of activity in the absence of inhibitor at the same micelle concentration. Solid white, 0.4 mol% R59949; solid gray, 2 mol% R59949; hatched white, 2 mol% R59022; and hatched gray, 8 mol% R59022.

activation of DGKα could also be demonstrated in the Triton/C16SB assay, albeit to a smaller extent than in octylglucoside/PS. Triton/C16SB mixed micelles readily solubilized R59949 (up to 2 mol%) and R59022 (up to 8 mol%). DGK inhibition by these agents was demonstrated readily in this assay. As R59949 and R59022 are lipophilic, inhibition was dependent on the mole fraction of inhibitor in the mixed micelles and not on its bulk solution concentration (Fig. 2). Inhibition of seven DGKs by R59949 in the Triton/C16SB assay is shown in Table 1 and Fig. 3. R59949 potently inhibited all of the Ca^{2+}-activated DGKs (thymus A, testis cytosol, salivary cytosol, and 3T3 DGK B), whereas Ca^{2+}-independent DAG kinases were inhibited only weakly (Fig. 3). The small inhibition of Ca^{2+}-independent DGKs could reflect either a weak interaction with the enzyme or a nonspecific effect on the assay. Similar results were obtained in the Triton/OTAC assay (Table 1). R59022 also inhibited Ca^{2+}-activated DGKs, but was less selective than R59949 (Table 1). Sensitivity of Ca^{2+}-activated DGKs to these agents did not correlate with their extent of stimulation by Ca^{2+} (Table 1). For example, 3T3 DGK B, which showed the smallest Ca^{2+} effect, was the most sensitive to both inhibitors. R59949 inhibited all four Ca^{2+}-activated DGKs 3-fold more potently than R59022 (data not shown). This ratio correlates well with the relative *in vivo* potency of these agents [12, 13].

Calcium-activated DGKs share Ca^{2+}-binding EF hand motifs in their regulatory domains [1, 2]. The observation

TABLE 1. Inhibition of DGKs by R59949 and R59022

	Ca^{2+} stimulation	Percent inhibition			
		R59022 (8 mol%)		R59949 (2 mol%)	
	Fold	C16SB assay	OTAC assay	C16SB assay	OTAC assay
Calcium-activated DGKs					
DGKα (thymus A)	80	69.9	68.2	79	77.4
Testis cytosol DGK	6.1	61.3	64.7	69	79.9
Salivary cytosol DGK	2.7	66.5	58.8	74	68.4
3T3 DGK B	1.6*	92.8	92.7	94.6	93.8
Calcium-independent DGKs					
3T3 DGK A	0.95	26.5	32.8	18	30.4
Thymus B DGK	0.95	42.5	29.2	18	13.2
Arachidonoyl DGK	1.0	32.7	35.4	21.7	24.4

Inhibition was examined in the Triton/C16SB and Triton/OTAC assays. R59022 was used at 8 mol% and R59949 at 2 mol%. Activities are expressed as percentages of activity in the absence of inhibitor. Calcium stimulation was measured using the octylglucoside/PS assay and is expressed as the ratio of activity in the presence of 50 μM Ca^{2+} to activity in the absence of Ca^{2+}. The octylglucoside/PS assay is more sensitive than Triton/C16SB to Ca^{2+} stimulation. Additional details of the assay procedures are given in Materials and Methods. Duplicate DGK assays were performed in each of two or more separate experiments. Specific activities (Triton/C16SB assay, nmol/min/mg) of the DGK preparations used were as follows: DGKα, 17.6; testis cytosol DGK, 0.57; salivary cytosol DGK, 0.16; 3T3 DGK B, 1.82; 3T3 DGK A, 0.13; thymus B DGK, 315; arachidonoyl DGK, 4.28.

* Calcium stimulation of 3T3 DGK B was assayed on two separate occasions. Stimulation observed was 1.57- and 1.72-fold. In both experiments, half-maximal stimulation of 3T3 DGK B occurred at 3 μM Ca^{2+}, which is similar to other Ca^{2+}-stimulated isoforms.

that R59949 specifically inhibited Ca^{2+}-activated DGKs could thus reflect an interaction with the EF hand region. In this case, Ca^{2+} might compete with binding of these agents to the enzyme. As shown in Fig. 4, however, R59949 inhibition of DGKα (thymus A) in the Triton/C16SB assay was independent of Ca^{2+}. While not precluding binding of the inhibitors to the EF hands, these results suggest that there is little interaction with Ca^{2+} binding. Direct evidence that R59949 does not interact with EF hands is presented below.

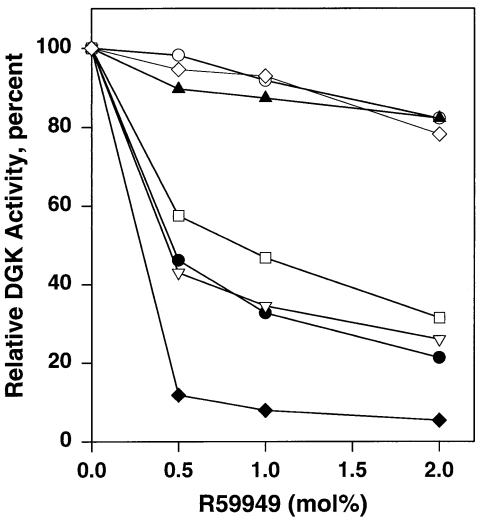


FIG. 3. Inhibition of DGK isoenzymes by R59949. DGK isoenzymes were prepared as described in Materials and Methods and examined for R59949 inhibition in the Triton/C16SB assay. Activities are expressed as percentages of the activity in the absence of inhibitor. Duplicate assays were performed in each of two separate experiments. Specific activities of the DGK preparations used are given in the legend of Table 1. The DGK isoforms examined include: (●) thymus A DGK (DGKα), (○) thymus B DGK, (□) testis cytosolic DGK, (▲) 3T3 DGK A, (◆) 3T3 DGK B, ▽ salivary cytosolic DGK, and (◇) arachidonoyl-DGK.

Interaction of R59949 with the DGKα Catalytic Domain

To map the site of R59949 interaction with DGKs, we transiently expressed full-length DGKα and two regulatory domain truncation mutants in COS-1 cells. Structures of these constructs are shown in Fig. 5. Consistent with previous results [6], DGKα was Ca^{2+}-activated. We also expressed a Ca^{2+}-independent isoform, DGKζ [30]. Cytosol fractions from COS-1 cells expressing these DGKs were assayed in Triton/C16SB. COS-1 cells express an endoge-

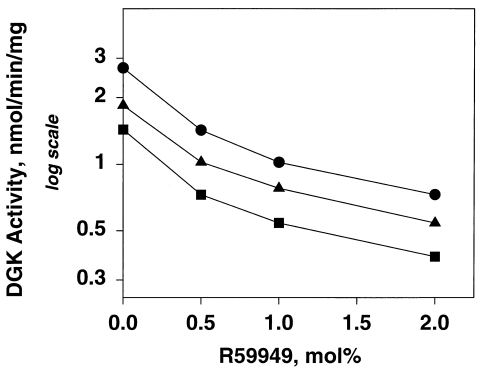


FIG. 4. Effect of Ca^{2+} on R59949 inhibition of DGKα. Inhibition of DGKα by R59949 was assessed in the Triton/C16SB assay at 0, 10, and 30 μM free Ca^{2+}. DGKα was prepared from thymus as described in Materials and Methods. The data shown are averages of duplicate determinations. Identical results were obtained with an independent DGKα preparation. Key: (■) no Ca^{2+}, (▲) 10 μM Ca^{2+}, and (●) 30 μM Ca^{2+}.

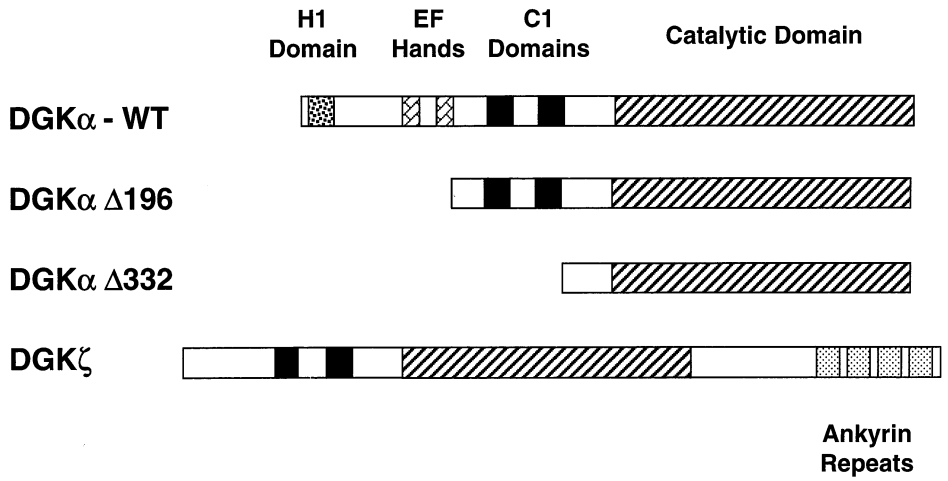


FIG. 5. Structures of DGKα truncation mutants used in this work. Schematic structures of wild-type DGKα and the Δ196 and Δ332 truncation mutants are shown. DGKζ, which was used as a control, is also shown.

nous DGK activity. However, transient expression of full-length DGKα or DGKα Δ196 resulted in marked elevation of total DGK activity. The Δ332 mutant expressed much lower activity, only 3-fold over the COS-1 background. DGKα activity expressed in COS cells was inhibited dramatically by R59949 (Fig. 6A). In contrast, DGKζ, a Ca^{2+}-independent isoform, was inhibited only slightly by the highest concentrations of inhibitor employed. We also examined DGKα Δ196, which lacks the EF hands (Fig. 5). As was noted earlier, this mutant was no longer stimulated by Ca^{2+} [6]. However, R59949 inhibited DGKα Δ196 to the same extent as wild-type DGKα (Fig. 6A). Thus,

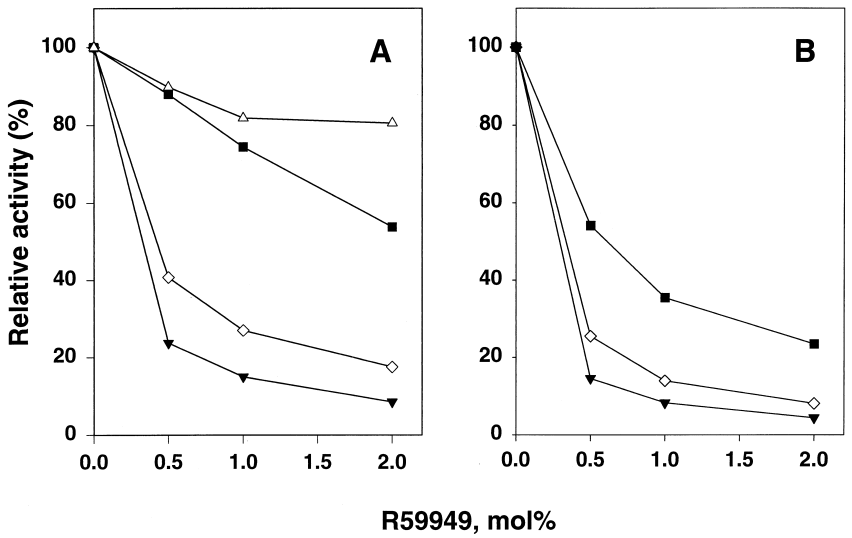


FIG. 6. R59949 inhibition of truncated DGKs. (A) Inhibition of DGK activities in COS cells. Extracts of COS-1 cells expressing wild-type DGKα, its truncation mutants, or wild-type DGKζ were assayed in Triton/C16SB as described in Materials and Methods. Activities were corrected for the endogenous COS-1 background by subtracting the activity of an extract of cells transfected with only the pSRE vector and assayed under identical conditions. In the absence of inhibitor, this background was equal to 1/3 of the total activity for the Δ332 mutant, which expressed the lowest activity of the constructs examined. (B) Inhibition of activities in yeast. Extracts of yeast expressing wild-type DGKα or its truncated mutants were prepared and purified as described in Materials and Methods. Then inhibition by R59949 was examined in the Triton/C16SB assay as before. *S. cerevisiae* does not express an endogenous DGK, and yeast transformed with the parent pYCDE2 vector did not express DGK activity that could be detected with any assay used in this work (data not shown). Background counts in assays of yeast not expressing a DGK activity were less than 2% of the activity expressed by the Δ332 mutant, which expressed the lowest activity of the constructs examined. As such, no background correction was required. All data are expressed as percentages of the activity in the absence of inhibitor. Specific activities (Triton C16SB assay, nmol/min/mg) of the COS-1 cell preparations were: wild-type DGKα, 5.02; DGKα Δ196, 33.3; DGKα Δ332, 0.82; and DGKζ, 1.55. These activities have all been corrected for a COS-1 background DGK activity of 0.23. Specific activities (Triton/C16SB assay, nmol/min/mg) of the yeast preparations were: wild-type DGKα, 810; DGKα Δ196, 54; and DGKα Δ332, 0.013. The relatively high specific activity of the yeast wild-type DGKα preparation reflects the fact that it was more purified than Δ196 or Δ332, the truncated constructs having proved unstable after the first chromatographic step. All data shown are averages of duplicate activity determinations, and all results have been confirmed in two additional enzyme preparations. Symbols: (◇) DGKα, (▼) DGKα Δ196, (■) DGKα Δ332, and (Δ) DGKζ.

although R59949 is selective for Ca^{2+}-activated DGKs, it does not appear to interact with the EF hands directly. This is consistent with our observation that inhibition was not competitive with Ca^{2+} (Fig. 4). We also examined DGKα Δ332, which lacks both the EF hands and the C1 domains. This mutant was also inhibited, but to a smaller extent than wild-type DGKα. Inhibition of the truncated DGKs in the Triton/OTAC assay was essentially the same as that in Triton/C16SB (data not shown).

Activity expressed by the Δ332 mutant was quite low. In fact, because of the endogenous COS cell activity, it has been controversial whether this mutant possesses DGK activity at all [34]. To exclude interference from the COS-1 background, we expressed the mutant DGKs in *S. cerevisiae*. This yeast does not have an endogenous DGK, and we did not detect DGK activity in yeast transformed with the pYCDE2 vector. All the constructs, including the Δ332 mutant, expressed DGK activity in yeast, and all of these activities were inhibited by R59949 (Fig. 6B). As in the COS-1 cells, the Δ332 mutant expressed only low activity. As with the COS-1 activities, full-length and Δ196 DGK activities were inhibited more potently than Δ332. Relatively weak inhibition of the Δ332 mutant expressed in COS cells could reflect a small error introduced during subtraction of the COS-1 background. Total DGK activity in COS-1 lysates transfected only with vector may not exactly reflect the background in cells expressing a DGK. The yeast results thus provide important confirmation of the findings in COS-1 cells. Inhibition of the Δ332 mutant, which contains none of the regulatory domain, indicates that R59949 interacts directly with the DGK catalytic domain.

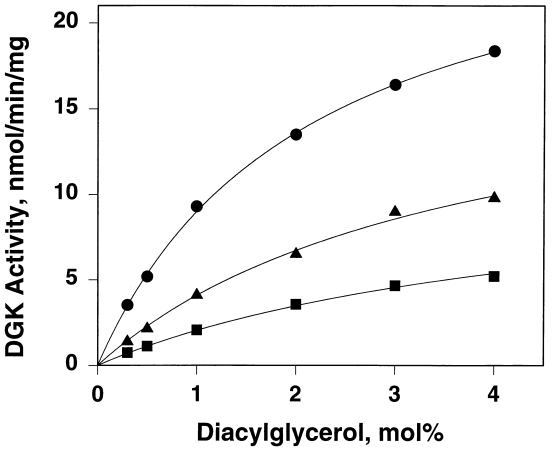


FIG. 7. Effect of R59949 on the DAG dependence of DGKα. The dependence of DGKα on 16:0,18:1-DAG is shown. Assays were performed in Triton/C16SB micelles. DGKα was purified from thymus. DAG was varied from 0.3 to 4.0 mol%, and MgATP was kept at 0.1 mM. Assays were performed at 0, 0.5, and 2.0 mol% R59949. The curves show predicted activities from the rate equation for partial mixed inhibition using constants given in the text. All data shown are averages of duplicate DGK activity measurements. Key: (●) no R59949, (▲) 0.5 mol% R59949, and (■) 2.0 mol% R59949.

Synergy of R59949 Inhibition with MgATP

Effects of R59949 on the substrate kinetics of DGKα were examined in the Triton C16SB assay. R59949 is a partial inhibitor of DGKs (Fig. 3). Figures 7 and 8 show dependencies of wild-type DGKα activity on DAG and MgATP at increasing concentrations of R59949. For each substrate, the inhibitor changed both V_m and the apparent K_m, indicating a mixed mechanism. Assuming random equilibrium substrate binding, which we have shown previously [25], and fitting these initial rates to the equation for partial mixed inhibition (given below) indicated an apparent K_i for R59949 binding to free enzyme of 3.2 ± 0.1 mol% [35]. In the absence of inhibitor, K_s(DAG) and K_s(MgATP) were 2.1 ± 0.1 mol% and 49 ± 3 μM, respectively. R59949 dramatically increased the apparent affinity for MgATP, but had only a small effect on DAG kinetics. The corresponding constants for the enzyme–R59949 complex were: K_{si}(DAG), 7.1 ± 1.0 mol%, and K_{si}(MgATP), 2.5 ± 0.2 μM. From these data, the constant for R59949 binding to enzyme–MgATP can be calculated to be 0.16 ± 0.02 mol%. As such, R59949 must bind the enzyme–MgATP complex 20-fold more tightly than free enzyme. This synergy of MgATP and R59949 binding is evident in Fig. 8 as a marked left-ward shift of the half-maximal velocity as inhibitor is increased. In fact, at MgATP concentrations of less than 10 μM, 2 mol% R59949 caused only 15% inhibition (Fig. 8). This small effect is equal to the weak inhibition seen with Ca^{2+}-independent DGKs at 2 mol% R59949 (Figs. 3 and 6).

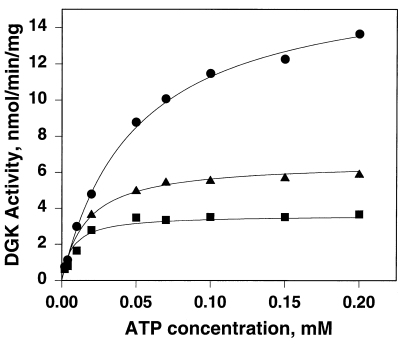


FIG. 8. Effect of R59949 on the MgATP dependence of DGKα. The dependence of thymus DGKα activity on MgATP is shown. Assays were performed in Triton/C16SB micelles. DGKα was purified from thymus. MgATP was varied from 2 to 200 μM and DAG was kept at 2.0 mol%. Assays were performed at 0, 0.5, and 2.0 mol% R59949. The curves show predicted activities from the rate equation for partial mixed inhibition using constants given in the text. All data shown are averages of duplicate DGK activity measurements. Loss of R59949 inhibition at low concentrations of MgATP, which is indicative of binding synergy, was confirmed in an independent experiment. Key: (●) no R59949, (▲) 0.5 mol% R59949, and (■) 2.0 mol% R59949.

The rate equation for partial mixed inhibition with random order equilibrium substrate addition that was used for curve fitting is given by:

$$v = \frac{V_m \cdot [\mathrm{DAG}] \cdot [\mathrm{MgATP}] + \frac{V_{mi} \cdot K_s(\mathrm{DAG}) \cdot K_s(\mathrm{MgATP})}{K_i \cdot K_{si}(\mathrm{DAG}) \cdot K_{si}(\mathrm{MgATP})} \cdot [I] \cdot [\mathrm{DAG}] \cdot [\mathrm{MgATP}]}{(K_s(\mathrm{DAG}) + [\mathrm{DAG}]) \cdot (K_s(\mathrm{MgATP}) + [\mathrm{MgATP}]) + K_s(\mathrm{DAG}) \cdot K_s(\mathrm{MgATP}) \cdot \frac{[I]}{K_i} \cdot \left(1 + \frac{[\mathrm{DAG}]}{K_{si}(\mathrm{DAG})}\right) \cdot \left(1 + \frac{[\mathrm{MgATP}]}{K_{si}(\mathrm{MgATP})}\right)}$$

V_m is the maximum velocity in the absence of inhibitor; V_{mi} is the maximum velocity in the presence of saturating inhibitor, K_s(DAG) and K_s(MgATP) are Michaelis constants in the absence of inhibitor; K_{si}(DAG) and K_{si}(MgATP) are Michaelis constants in the presence of saturating inhibitor; and K_i is the enzyme-inhibitor dissociation constant. The dissociation constant of inhibitor from the DGKα/MgATP complex is then equal to $K_i \cdot K_{si}$(MgATP)/K_s(MgATP). To avoid an excessive number of independent variables, V_m, K_s(DAG), and K_s(MgATP) were determined first by fitting the data obtained in the absence of inhibitor and then fixed in the equation prior to determination of V_{mi}, K_i, K_{si}(DAG), and K_{si}(MgATP).

DISCUSSION

This work demonstrates that R59949 and R59022 interact directly with DGKs. A few studies have examined highly purified DGK preparations for *in vitro* inhibition by these agents. R59022 inhibited DGKα assayed in the presence of PS or sphingosine [21]. In this same study, another DGK prepared from thymus was not inhibited. In a Triton micelle assay, neither R59022 nor R59949 inhibited an arachidonoyl DAG-specific DGK from testis [22]. R59022 inhibited three platelet DGK activities from 20 to 80% in a deoxycholate assay [23]. A number of studies have also examined less purified DGK preparations. In a deoxycholate-based assay, R59022 inhibited the DGK activity of rat liver nuclei, but not microsomes [36], and partially inhibited activities in membrane and cytosol fractions of fMetLeuPhe-stimulated neutrophils [37]. In octylglucoside/PS micelles, R59022 and R59949 partially inhibited DGKε expressed in COS-7 cells [38], but had no effect on DGKζ [39]. In this same assay, R59022 inhibited a DGK activity extracted from guinea pig taenia coli [15] but failed to inhibit two activities purified from rat brain [40]. Many of these studies utilized anionic DGK activators, such as PS and deoxycholate. Intramicellar complexation with cationic moieties on R59022 and R59949 may reverse activation by anionic lipids and would be difficult to distinguish from direct enzyme inhibition. We have also noted these agents to be poorly soluble in octylglucoside and deoxycholate. Another complicating factor is that some DGKs are activated only weakly, or even inhibited, by anionic lipids [25]. The Triton/C16SB assay overcomes these problems and has the additional advantage of yielding high activities with all DGK isoforms. Our results indicate that R59949 and R59022 inhibit some DGK subtypes in direct proportion to their *in vivo* potency. These results thus confirm that DGKs are direct targets of R59022 and R59949.

R59949 was selective among DGKs. The four Ca^{2+}-activated DGKs examined were all sensitive to this agent, while the three Ca^{2+}-insensitive isoforms exhibited only small effects. Future *in vivo* studies will thus need to demonstrate that the DGK being examined is sensitive to the agent employed. Examination of a series of DGKα deletion mutants indicated that inhibition does not require EF hand motifs unique to Ca^{2+}-activated DGKs. Rather, R59949 appears to interact directly with the catalytic domain. The strong synergy of inhibitor and MgATP binding also is consistent with a catalytic domain interaction. Given these findings, the apparent selectivity of R59949 for Ca^{2+}-activated DGKs probably reflects the fact that catalytic domain sequences of EF-hand-containing DGKs exhibit much stronger homology to each other than to other DGK isoforms.* Inhibition of the Δ332 mutant, expressed as a percent of basal activity, was less than that of the other constructs. This mutant lacks the C1 domains. In PKCγ and Raf-1, C1 domains appear to play a role in targeting to membranes [5]. The comparatively low *in vitro* activity expressed by this mutant may thus reflect an impairment of surface targeting. This may alter the kinetics in such a way as to render the residual activity less sensitive to the inhibitor. For example, by targeting the enzyme to micelles, the C1 domains may facilitate transfer of inhibitor from micelles to a site on the catalytic domain. The Ca^{2+}-activated DGKs examined exhibited wide variability in their sensitivity to Ca^{2+}, which did not correlate with their inhibitor sensitivity. Modest Ca^{2+} stimulation of activity does not preclude an important role for Ca^{2+} in regulating these DGKs. The assays utilized in this work

* Conserved DGK catalytic domain sequences were aligned in PSI-BLAST searches using the BLOSUM62 matrix [41]. Homologies between EF-hand-containing DGKs, including a predicted sequence from *Caenorhabditis elegans*, were all 78% or greater. Homologies between EF-hand-containing and other DGKs were all 70% or less. Alignments of DGK catalytic domains can also be viewed at the Pfam web server, http://www.sanger.ac.uk/Software/Pfam/, under the domain names DAGKc and DAGKa.

measure only DAG phosphorylation. *In vivo*, Ca^{2+} may regulate events not detected by these assays, such as protein–protein interactions involved in targeting an activated DGK to its substrate [9, 42].

A surprising finding was the marked potentiation of R59949 inhibition by MgATP. Plausible mechanisms for an R59949 interaction with MgATP binding would include binding to the catalytic domain in proximity to the MgATP site or a conformational change of the enzyme that exposes both MgATP and R59949 binding sites. With regard to *in vivo* inhibition of DGKα, the MgATP concentration in animal cells is 2–5 mM, over an order of magnitude greater than its apparent K_s. It is thus likely that, *in vivo*, R59949 binds to the enzyme–MgATP complex. Inasmuch as detergent mixed micelles and membrane bilayers are highly non-ideal systems, the inhibition parameters in this study cannot be directly related to *in vivo* affinities. Rather the utility of the mixed micelle assay is in permitting steady-state kinetic analyses with these inhibitors. The observation that the R59949 is 3-fold more potent than R59022 correlates well with the *in vivo* potency of these agents and strongly supports the hypothesis that the *in vitro* inhibition observed reflects the *in vivo* mechanism.

This work was performed while Dr. James P. Walsh was a Pfizer Scholar. Additional support was provided by a grant from the US Veterans Health Administration Medical Research Service to Dr. James P. Walsh. We thank Drs. Tom Kovala and Anna Depaoli-Roach for assistance with transient expression of DGKs in COS-1 cells. We also thank Dr. Kaoru Goto for providing DGKζ cDNA.

References

1. Kanoh H, Sakane F, Imai S and Wada I, Diacylglycerol kinase and phosphatidic acid phosphatase–enzymes metabolizing lipid second messengers. *Cell Signal* **5:** 495–503, 1993.
2. Sakane F and Kanoh H, Molecules in focus: Diacylglycerol kinase. *Int J Biochem Cell Biol* **29:** 1139–1143, 1997.
3. Topham MK and Prescott SM, Mammalian diacylglycerol kinases, a family of lipid kinases with signaling functions. *J Biol Chem* **274:** 11447–11450, 1999.
4. Flores I, Casaseca T, Martinez-A C, Kanoh H and Merida I, Phosphatidic acid generation through interleukin 2 (IL-2)-induced α-diacylglycerol kinase activation is an essential step in IL-2-mediated lymphocyte proliferation. *J Biol Chem* **271:** 10334–10340, 1996.
5. Hurley JH, Newton AC, Parker PJ, Blumberg PM and Nishizuka Y, Taxonomy and function of C1 protein kinase C homology domains. *Protein Sci* **6:** 477–480, 1997.
6. Sakane F, Imai S, Yamada K and Kanoh H, The regulatory role of EF-hand motifs of pig 80K diacylglycerol kinase as assessed using truncation and deletion mutants. *Biochem Biophys Res Commun* **181:** 1015–1021, 1991.
7. Yamada K and Sakane F, The different effects of sphingosine on diacylglycerol kinase isozymes in Jurkat cells, a human T-cell line. *Biochim Biophys Acta* **1169:** 211–216, 1993.
8. van der Bend RL, de Widt J, Hilkmann H and van Blitterswijk WJ, Diacylglycerol kinase in receptor-stimulated cells converts its substrate in a topologically restricted manner. *J Biol Chem* **269:** 4098–4102, 1994.
9. Montgomery RB, Moscatello DK, Wong AJ and Stahl WL, Epidermal growth factor receptor stimulation of diacylglycerol kinase. *Biochem Biophys Res Commun* **232:** 111–116, 1997.
10. Wada I, Kai M, Imai S, Sakane F and Kanoh H, Translocation of diacylglycerol kinase α to the nuclear matrix of rat thymocytes and peripheral T-lymphocytes. *FEBS Lett* **393:** 48–52, 1996.
11. Nobe K, Ohata H and Momose K, Receptor-mediated diacylglycerol kinase translocation dependent on both transient increase in the intracellular calcium concentration and modification by protein kinase C. *Biochem Pharmacol* **53:** 1683–1694, 1997.
12. de Chaffoy de Courcelles DC, Roevens P and Van Belle H, R 59 022, a diacylglycerol kinase inhibitor. Its effect on diacylglycerol and thrombin-induced C kinase activation in the intact platelet. *J Biol Chem* **260:** 15762–15770, 1985.
13. de Chaffoy de Courcelles D, Roevens P, Van Belle H, Kennis L, Somers Y and De Clerck F, The role of endogenously formed diacylglycerol in the propagation and termination of platelet activation. A biochemical and functional analysis using the novel diacylglycerol kinase inhibitor, R 59 949. *J Biol Chem* **264:** 3274–3285, 1989.
14. Tohmatsu T, Nakashima S, Hattori H, Suganuma A and Nozawa Y, A role of diacylglycerol kinase in stimulus-secretion coupling of human platelets. Dissociation of serotonin secretion from Ca^{2+} mobilization. *Thromb Res* **47:** 25–35, 1987.
15. Nobe K, Ohata H and Momose K, Activation of diacylglycerol kinase by carbachol in guinea pig taenia coli. *Biochem Pharmacol* **48:** 2005–2014, 1994.
16. Mege JL, Tao W, Molski TF, Gomez-Cambronero J, Huang CK, Becker EL and Sha'afi RI, Diacylglycerol kinase inhibitor R59022 and stimulated neutrophil responses. *Am J Physiol* **255:** C589–C594, 1988.
17. Ohtsuka T, Hiura M, Yoshida K, Okamura N and Ishibashi S, A diacylglycerol kinase inhibitor, R 59 022, potentiates superoxide anion production and 46-kDa protein phosphorylation in guinea pig polymorphonuclear leukocytes. *J Biol Chem* **265:** 15418–15423, 1990.
18. Keefe KL and Warner JA, Effect of R59022, an inhibitor of diacylglycerol kinase, on IgE-mediated histamine release from human lung mast cells and basophils. *Agents Actions* **43:** 1–6, 1994.
19. Ederveen AG, van Emst-de Vries SE, De Pont JJ and Willems PH, The diacylglycerol kinase inhibitor, R59022, potentiates cholecystokinin-induced enzyme secretion from rabbit pancreatic acini. *Eur J Biochem* **188:** 333–338, 1990.
20. Inui H, Kondo T, Konishi F, Kitami Y and Inagami T, Participation of diacylglycerol kinase in mitogenic signal transduction induced by platelet-derived growth factor in vascular smooth muscle cells. *Biochem Biophys Res Commun* **205:** 1338–1344, 1994.
21. Sakane F, Yamada K and Kanoh H, Different effects of sphingosine, R59022 and anionic amphiphiles on two diacylglycerol kinase isozymes purified from porcine thymus cytosol. *FEBS Lett* **255:** 409–413, 1989.
22. Walsh JP, Suen R, Lemaitre RN and Glomset JA, Arachidonoyl-diacylglycerol kinase from bovine testis. Purification and properties. *J Biol Chem* **269:** 21155–21164, 1994.
23. Yada Y, Ozeki T, Kanoh H and Nozawa Y, Purification and characterization of cytosolic diacylglycerol kinases of human platelets. *J Biol Chem* **265:** 19237–19243, 1990.
24. Walsh JP and Bell RM, Diacylglycerol kinase from *Escherichia coli*. *Methods Enzymol* **209:** 153–162, 1992.
25. Walsh JP, Suen R and Glomset JA, Arachidonoyl-diacylglycerol kinase: Specific *in vitro* inhibition by polyphosphoinositides suggests a mechanism for regulation of phosphatidylinositol biosynthesis. *J Biol Chem* **270:** 28647–28653, 1995.
26. Goto K, Watanabe M, Kondo H, Yuasa H, Sakane F and

Kanoh H, Gene cloning, sequence, expression and *in situ* localization of 80 kDa diacylglycerol kinase specific to oligodendrocyte of rat brain. *Brain Res Mol Brain Res* **16:** 75–87, 1992.
27. 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.
28. Walsh JP and Bell RM, *sn*-1,2-Diacylglycerol kinase of *Escherichia coli*, Mixed micellar analysis of the phospholipid cofactor requirement and divalent cation dependence. *J Biol Chem* **261:** 6239–6247, 1986.
29. Sakane F, Kai M, Wada I, Imai S and Kanoh H, The C-terminal part of diacylglycerol kinase α lacking zinc fingers serves as a catalytic domain. *Biochem J* **318:** 583–590, 1996.
30. Goto K and Kondo H, A 104-kDa diacylglycerol kinase containing ankyrin-like repeats localizes in the cell nucleus. *Proc Natl Acad Sci USA* **93:** 11196–11201, 1996.
31. Chen C and Okayama H, High efficiency transformation of mammalian cells by plasmid DNA. *Mol Cell Biol* **7:** 2745–2752, 1987.
32. Hadfield C, Cashmore AM and Meacock PA, An efficient chloramphenicol-resistance marker for *Saccharomyces cerevisiae* and *Escherichia coli*. *Gene* **45:** 149–158, 1986.
33. Schena M, Picard D and Yamamoto KR, Vectors for constitutive and inducible gene expression in yeast. *Methods Enzymol* **194:** 389–398, 1991.
34. van Blitterswijk WJ and Houssa B, Diacylglycerol kinases in signal transduction. *Chem Phys Lipids* **98:** 95–108, 1999.
35. Frieden C, Treatment of enzyme kinetic data. *J Biol Chem* **239:** 3522–3531, 1964.
36. Previati M, Bertagnolo V, Mazzoni M, Osti F, Borgatti P and Capitani S, Diacylglycerol kinase activity in rat liver nuclei. *Cell Signal* **6:** 393–403, 1994.
37. Hurttia H and Leino L, Subcellular localization of diacylglycerol kinase activity in stimulated and unstimulated human peripheral blood lymphocytes and neutrophils. *Biochem Mol Biol Int* **40:** 579–585, 1996.
38. Tang W, Bunting M, Zimmerman GA, McIntyre TM and Prescott SM, Molecular cloning of a novel human diacylglycerol kinase highly selective for arachidonate-containing substrates. *J Biol Chem* **271:** 10237–10241, 1996.
39. Bunting M, Tang W, Zimmerman GA, McIntyre TM and Prescott SM, Molecular cloning and characterization of a novel human diacylglycerol kinase ζ. *J Biol Chem* **271:** 10230–10236, 1996.
40. Chen Q, Klemm N and Jeng I, Characterization of two cytosolic diacylglycerol kinase forms. *J Neurochem* **60:** 1212–1219, 1993.
41. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W and Lipman D, Gapped BLAST and PSI-BLAST, A new generation of protein database search programs. *Nucleic Acids Res* **25:** 3389–3402, 1997.
42. Tolias KF, Couvillon AD, Cantley LC and Carpenter CL, Characterization of a Rac1- and RhoGDI-associated lipid kinase signaling complex. *Mol Cell Biol* **18:** 762–770, 1998.