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# Structure–Activity Relationship of Synthetic Branched-Chain Distearoylglycerol (Distearin) as Protein Kinase C Activators†

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ABSTRACT: Several representative branched-chain analogues of distearin (DS) were synthesized and tested for their abilities to activate protein kinase C (PKC) and to compete for the binding of [$^3$H]phorbol 12,13-dibutyrate (PDBu) to the enzyme. Substitutions of stearoyl moieties at *sn*-1 and *sn*-2 with 8-methylstearate decreased activities on these parameters, relative to those of the parental diacylglycerol DS, a weak PKC activator. Substitutions with 8-butyl, 4-butyl, or 8-phenyl derivatives, on the other hand, increased activities of the resulting analogues to levels comparable to those seen for diolein (DO), a diacylglycerol prototype shown to be a potent PKC activator. Kinetic analysis indicated that 8-methyldistearin (8-MeDS) acted by decreasing, whereas 8-butyldistearin (8-BuDS) and 8-phenyldistearin (8-PhDS) acted by increasing, the affinities of PKC for phosphatidylserine (PS, a phospholipid cofactor) and $Ca^{2+}$ compared to the values seen in the absence or presence of DS. The stimulatory effect of 8-BuDS and 8-PhDS on PKC, as DO, was additive to that of 1,2-(8-butyl)distearoylphosphatidylcholine [1,2(8-Bu)DSPC] and, moreover, they abolished the marked inhibition of the enzyme activity caused by high concentrations of 1,2(8-Bu)DSPC. The present findings demonstrated a structure–activity relationship of the branched-chain DS analogues in the regulation of PKC, perhaps related to their abilities to specifically modify interactions of PKC with PS and/or $Ca^{2+}$ critically involved in enzyme activation/inactivation.

Earlier studies have shown that unsaturated long-chain fatty acids at *sn-1* and/or *sn-2* of glycerol are required for diacylglycerol derivatives to be PKC[1] activators (Kishimoto et al., 1980; Mori et al., 1982). For example, diarachidonin (di 20:4), DO (di 18:1), and 1-stearoyl-2-oleoylglycerol (18:0; 18:1) are active but DS (di 18:0) or dipalmitin (di 16:0) are less active. The cell-permeable diacylglycerol, 1-oleoyl-2-acetylglycerol, is also active (Fujita et al., 1984), indicating that presence of a short-chain acyl moiety does not impair the

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[1] Abbreviations: PKC, protein kinase C; DO, diolein; DS, distearin; PS, phosphatidylserine; PC, phosphatidylcholine; DSPC, 1,2-distearoylphosphatidylcholine; 1,2(8-Bu)DSPC, *sn*-1,2-(8-butyl)DSPC; PDBu, phorbol 12,13-dibutyrate; TLC, thin-layer chromatograph. The methyl (Me), butyl (Bu), and phenyl (Ph) analogues of branched-chain DS are abbreviated as follows: for example, 8-MeDS and 8-BuDS indicate that stearoyl moieties at *sn-1* and *sn-2* in DS were substituted by 8-methylstearic acid and 8-butylstearic acid, respectively (see Figure 1 for structures).

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activity of the synthetic analogue. More recent studies have extended and modified the structural specificity of diacylglycerols as PKC activators: Synthetic, permeable diacylglycerols containing saturated short-chain fatty acids, such as dioctanoylglycerol (di 8:0), are fully active (Lapetina et al., 1985; Ganong et al., 1986).

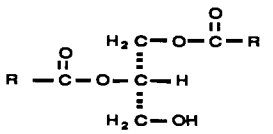
We reported recently that the activity of DSPC, a weak inhibitor of PKC, can be markedly modified by substitutions of stearic acid moieties at *sn-1* and/or *sn-2* with various branched-chain analogues (Charp et al., 1988b). For example, replacements of stearic acids at *sn-1* and *sn-2* by the 16-methyl derivative greatly increase its inhibitory potency, but replacements by 8-butyl and 8-phenyl derivatives conversely transform the resulting DSPC analogues into potent activators of the enzyme. We wondered whether a similar structure–activity relationship might exist for the branched-chain analogues of DS, a weak PKC activator. Indeed, we found in the present studies that substituents on the stearic moiety could markedly increase or decrease the abilities of the resulting DS analogues to activate PKC.

## Experimental Procedures

*Materials.* 3-Benzyl-*sn*-glycerol, DO, DS, PS (bovine brain), histone H1, and PDBu were purchased from Sigma Chemical Co. (St. Louis, MO); TLC sheets precoated with a 254-nm fluorescent indicator and all other starting materials and reagents were from Aldrich Chemical Co. (Milwaukee, WI); Silicar silica gel (100–200 mesh) was from Mallinckrodt Chemicals (St. Louis, MO); [$^3$H]PDBu (15.8 Ci/mol) was from Du Pont/New England Nuclear (Albany, MA).

*Synthesis of Branched-Chain DS Analogues.* The procedures for synthesis of branched-chain analogues of stearic acid (Cason et al., 1949; Charp et al., 1988b) and diacylglycerol derivatives (Hibl et al., 1983) were as described. The synthesis of 8-BuDS will be described here briefly as an example. To a mixture of 3-benzyl-*sn*-glycerol (114 mg, 0.63 mmol), 8-butylstearic acid (429 mg, 1.3 mmol), and 4-(methylamino)pyridine (62 mg, 0.51 mmol) dissolved in 5 mL of $CHCl_3$ (freshly distilled over $P_2O_5$) was added 260 mg (1.3 mmol) of dicyclohexylcarbodiimide. The resulting solution was then stirred with a Teflon-coated magnetic stirring bar for 6 days. The product was purified by column chromatography with the use of 5 g of Silicar silica gel. The eluting solvents were as follows: (1) 40 mL of hexanes; (2) 200 mL of hexanes/diisopropyl ether (40:1 v/v); and (3) 200 mL of hexanes/diisopropyl ether (20:1 v/v). The procedure afforded 0.51 mg (98% yield) of 1,2-bis(*dl*-8-butylstearoyl)-3-benzyl-*sn*-glycerol, which was subsequently dissolved in 10 mL of anhydrous tetrahydrofuran. Catalytic hydrogenolysis of the benzyl group was accomplished in the presence of 0.3 g of 10% Pd on carbon for 6 h under Parr conditions. After tetrahydrofuran was distilled, the product, 8-BuDS, was twice purified by column chromatography with the use of 6.5 g of silica gel. The eluting solvents were (1) 60 mL of hexanes/diisopropyl ether (10:1 v/v) and (2) 200 mL of hexanes/diisopropyl ether (1:1 v/v). This procedure afforded 503 mg (73% yield) of 8-BuDS. The purity of this and other branched-chain analogues of DS (Figure 1) was verified by TLC, proton NMR spectra (obtained on a Nicolet FT 360-MHz spectrometer or General Electric QE 300-MHz spectrometer) and $^{13}$C NMR decoupled spectra (obtained with General Electric QE-300 MHz spectrometer or Bruker WP-200SY spectrometer). All analytical data were consistent with the expected structures of the DS analogues.

*PKC Assay and [$^3$H]PDBu Binding.* PKC was purified through the step of phenyl-Sepharose chromatography (Wise et al., 1982). The enzyme activity was assayed under modified conditions (Charp et al., 1988b) using suboptimal concentrations of PS and $CaCl_2$ for the purpose of maximizing the stimulatory effect of diacylglycerol or phorbol ester. Briefly, the reaction mixtures (0.2 mL) contained 5 μmol of Tris-HCl (pH 7.5), 2 μmol of $MgCl_2$, 2 μg of PS, 40 μg of histone H1, 2 nmol of $CaCl_2$, 1.6 nmol of [$\gamma$-$^{32}$P]ATP (containing about $1.5 \times 10^6$ cpm), and various concentrations of diacylglycerols, as indicated. DO, DS, and DS analogues were mixed with PS in chloroform/methanol (1:1 v/v) at desired concentrations and dried under a stream of $N_2$. The dried lipids were resuspended in 20 mM Tris-HCl (pH 7.5), vortexed, and then sonicated by using a sonifier cell disruptor (Heat System-Ultrasonic Inc.) with a microtip (Plainview, Long Island, NY) set at a 60% power output for 30 s at 23 °C. The sonicated preparations of PS–diacylglycerols were clear at all concentrations and combinations of the lipids used. Free $Ca^{2+}$ concentrations (Figure 4) were estimated by the EGTA–$Ca^{2+}$ buffer system (Solaro & Shiner, 1976), without taking into account the concentrations of $Mg^{2+}$ and ATP and other reagents present in the incubation mixtures. The binding of [$^3$H]PDBu to PKC was carried out by the procedure of Sharkey and Blumberg (1985), but using a low concentration (8 instead of 100 μg/mL) of PS as we reported recently (Charp et al., 1988a). All experiments were performed in triplicate and repeated two or three times to ascertain the reproducibility of the findings. [$\gamma$-$^{32}$P]ATP was prepared as described by Post and Sen (1967).



| | |
|---|---|
| DS | $R = (CH_2)_{16}CH_3$ |
| 8-MeDS | $R = (CH_2)_6CH(Me)(CH_2)_9CH_3$ |
| 8-PhDS | $R = (CH_2)_6CH(Ph)(CH_2)_9CH_3$ |
| 8-BuDS | $R = (CH_2)_6CH(n\text{-}Bu)(CH_2)_9CH_3$ |
| 4-BuDS | $R = (CH_2)_2CH(n\text{-}Bu)(CH_2)_{13}CH_3$ |

FIGURE 1: Structures of DS analogues.

## Results

The structures of DS and its branched-chain analogues are shown in Figure 1. As reported previously (Kishimoto et al., 1980; Mori et al., 1982), DO was more potent than DS as a PKC activator (Figure 2), indicating a requirement of unsaturated long-chain fatty acid in diacylglycerol for its activity. The potency of DS, however, was increased when the stearic acid moieties at *sn-1* and *sn-2* were substituted with butyl or phenyl groups at the 4- or 8-position of the fatty acid. Thus, the resulting branched-chain analogues (4-BuDS, 8-BuDS, and 8-PhDS) were found to be nearly as potent as or even slightly more potent than DO, producing a half-maximal stimulation at about 0.1 mg/mL (Figure 2). A decreased potency, however, was noted for 8-MeDS, in which the stearic acid moieties were substituted with methyl groups at the 8-position. We reported recently a similar structure–activity relationship for the branched-chain analogues of DSPC in regulating PKC activity; the methyl DSPC analogues were inhibitors, whereas, in contrast, the butyl or phenyl DSPC analogues were activators of the enzyme (Charp et al., 1988b).

The effectiveness of DS and its analogues, compared to DO, in PKC activation as a function of PS (Figure 3) and $Ca^{2+}$ (Figure 4) was examined. The kinetic parameters from these and similar experiments were determined by using double-reciprocal analysis, and the results are summarized in Table I. It was clear that DS, 8-BuDS, and 8-PhDS, like DO, were

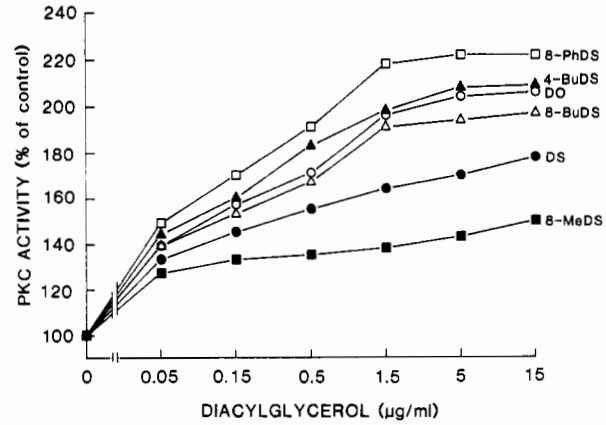



FIGURE 2: Comparative potency of DO, DS, and DS analogues on PKC activation. The enzyme was assayed under the modified conditions (i.e., 10 μg/mL of PS and 10 μM $CaCl_2$) in the presence of varying concentrations and different diacylglycerols, as indicated. The enzyme activity value (16.4 pmol/min) seen in the absence of added diacylglycerol was taken as 100%. The data presented are means of triplicate assays, with assay errors being less than 3%. Similar results were obtained in three separate sets of experiments.

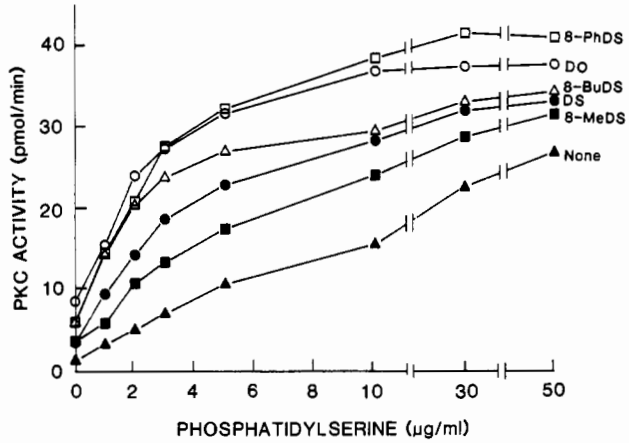



FIGURE 3: Comparative effectiveness of DO, DS, and DS analogues on PS-dependent PKC activation. The enzyme was assayed under the modified conditions in the presence of diacylglycerol (15 μg/mL), 10 μM $CaCl_2$, and varying concentrations of PS, as indicated. The data presented are means of triplicate assays, with assay errors being less than 5%. Similar results were obtained in three separate sets of experiments.

Table I: Summary of Kinetic Constants of PKC Activation by Diacylglycerols[a]

| | PS | | $Ca^{2+}$ | |
|---|---|---|---|---|
| diacylglycerol | $K_m$ (μg/mL) | $V_{max}$ (pmol/min) | $K_m$ (μM) | $V_{max}$ (pmol/min) |
| none (control) | 10.0 ± 1.2 | 31 ± 3 | 28.6 ± 1.2 | 36 ± 8 |
| DO | 1.6 ± 0.3[b] | 41 ± 2[b] | 0.9 ± 0.2[b] | 74 ± 9[b] |
| DS | 2.7 ± 0.5[b] | 35 ± 3 | 6.0 ± 0.2[b] | 57 ± 6[b] |
| 8-MeDS | 8.3 ± 1.3 | 34 ± 2 | 27.0 ± 0.8 | 56 ± 5[b] |
| 8-BuDS | 1.8 ± 0.2[b] | 40 ± 3[b] | 3.8 ± 1.1[b] | 67 ± 7[b] |
| 8-PhDS | 2.0 ± 0.3[b] | 44 ± 2[b] | 0.9 ± 0.3[b] | 77 ± 5[b] |

[a] The data presented are means ± SE of three experiments. [b] Significantly different from the control ($p < 0.05$).

able to markedly decrease $K_m$ values of PKC for PS and $Ca^{2+}$, whereas 8-MeDS was not effective. Small but significant increases (30–42%) in $V_{max}$ values determined as a function of PS were seen only for DO, 8-BuDS, and 8-PhDS. More pronounced increases (86–114%) in $V_{max}$ values were noted for DO, 8-BuDS, and 8-PhDS and, in addition, smaller increases (56–58%) were also noted for DS and 8-MeDS when the values were determined as a function of $Ca^{2+}$.

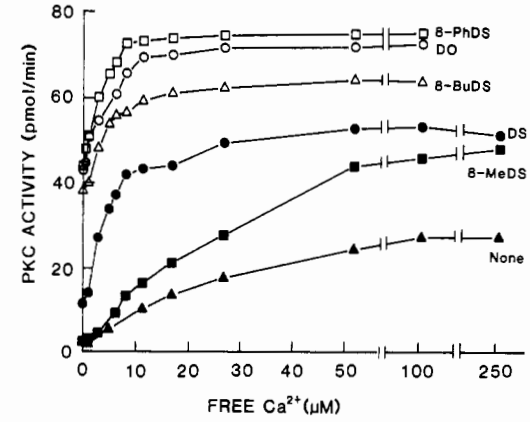



FIGURE 4: Comparative effects of DO, DS, and DS analogues on $Ca^{2+}$-dependent PKC activation. The enzyme was assayed under the modified conditions in the presence of diacylglycerol (15 μg/mL), 10 μg/mL PS, and varying concentrations of free $Ca^{2+}$, as indicated. The data presented are means of triplicate assays, with assay errors being less than 4%. Similar results were obtained in two separate sets of experiments.

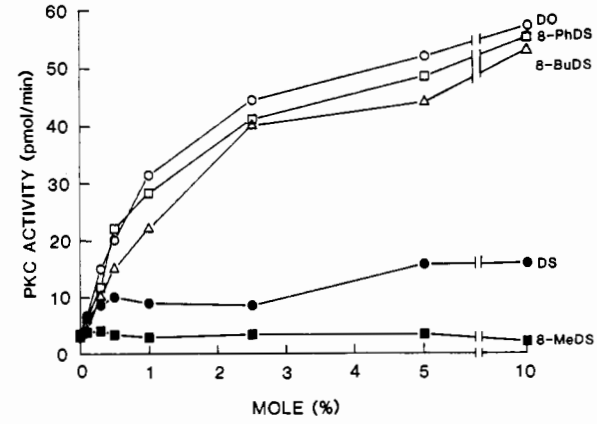



FIGURE 5: PKC activation by DO, DS, and DS analogues as assayed by using a mixed-micelle system, essentially according to the method of Hannun et al. (1986), which contained 0.3% Triton X-100, 6 mol % PS (8.4 molecules/micelle), 200 μM $CaCl_2$, and varying mole percent diacylglycerol, as indicated. The data presented are means of triplicate assays, with assay errors being less than 5%. Similar results were obtained in two separate sets of experiments. One mole percent diacylglycerol was calculated to be 1.4 molecules/micelle.

The relative effectiveness of the analogues was further investigated by using the Triton X-100 mixed-micelle system (Hannun et al., 1986). It was observed that 8-BuDS and 8-PhDS were essentially as active as DO in PKC activation, whereas DS was only moderately active and 8-MeDS was practically inactive (Figure 5). If one assumed 10 mol % DO, 8-BuDS, or 8-PhDS could fully activate PKC, a half-maximal stimulation by these agents was achieved at 1.0–1.5 mol %, calculated to be 1.5–2.0 molecules of diacylglycerol/micelle under the experimental conditions.

Next, we further investigated the activity of the analogues on the basis of competitive inhibition of [$^3$H]PDBu binding to PKC (Figure 6). The analogues 8-BuDS and 8-PhDS, similar to DO, produced a 50% inhibition of binding at about 0.1 μg/mL, corresponding to the value that gave a 50% stimulation of PKC activity as shown earlier in Figure 2. The 4-BuDS analogue was as active as 8-BuDS (data not shown). DS was much less active, whereas 8-MeDS was practically inactive (Figure 6).

We reported recently that 1,2-(8-Bu)DSPC stimulated PKC up to 3-fold at low concentrations but markedly inhibited it at high concentrations when the enzyme was assayed under the modified (suboptimal) conditions (Charp et al., 1988b).

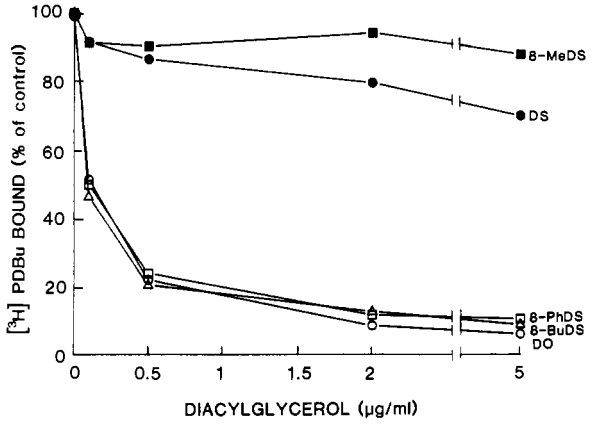



FIGURE 6: Comparative effects of DO, DS, and DS analogues on [$^3$H]PDBU binding to PKC. The enzyme (2 μg) was incubated for 30 min at 37 °C in the presence of 8 μg/mL PS, 1 μM $CaCl_2$, 16 nM [$^3$H]PDBU (17000 cpm), with or without 200 μM nonradioactive PDBU, and varying concentrations of diacylglycerols, as indicated. The nonspecific binding was less than 10% of total binding. The specific binding (75 pmol/mg of enzyme preparation) seen in the absence of diacylglycerol was taken as 100%. The data presented are means of triplicate assays, with assay errors being less than 5%. Similar results were obtained in three separate sets of experiments.

In the present studies, we noted that the stimulation seen with a fixed concentration (15 μg/mL) of DO, 8-BuDS, or 8-PhDS was additive to the stimulation afforded by increasing concentrations of 1,2-(8-Bu)DSPC (Figure 7A). Interestingly, the inhibitory effect of the DSPC analogue at high concentrations (>15 μg/mL) was completely abolished in the presence of each one of the diacylglycerols and, moreover, their stimulatory effect was unaffected by the inhibitory concentrations of the DSPC analogue. 8-MeDS, a poor activator, in comparison, was unable to abolish the PKC inhibition caused by 1,2-(8-Bu)DSPC (data not shown). Similar phenomena described above were also observed for the combinations of increasing concentrations of 8-BuDS and 1,2-(8-Bu)DSPC (Figure 7B), suggesting that the two classes of lipids might act differently in PKC activation.

## DISCUSSION

Although the detailed molecular mechanisms underlying PKC activation are still unclear, hydrophobic interactions of the enzyme protein with PS vesicles or micelles in the presence of $Ca^{2+}$ are clearly involved (Nishizuka, 1984; Turner & Kuo, 1985; Ganong et al., 1986). Diacylglycerol (Kishimoto et al., 1980) and phorbol ester (Castagna et al., 1982) activate the enzyme presumably by promoting such interactions, as suggested by their abilities to increase the affinity of the enzyme for PS and $Ca^{2+}$. Analogously, certain lipophilic agents, such as peptide antibiotic polymyxin B (Mazzei et al., 1982), antileukemic agent alkyllysophospholipid (Helfman et al., 1983), and antiestrogen tamoxifen (Su et al., 1984), inhibit PKC competitively with respect to PS, thus decreasing its affinity for the phospholipid cofactor. More recently, Ganong et al. (1986), on the basis of studies using various synthetic analogues of diacylglycerol containing saturated short-chain fatty acids and using the mixed-micelle assay system, proposed that PKC activation by diacylglycerol requires interactions of both carboxyl moieties of the oxygen esters of fatty acids and the 3-hydroxyl moiety of glycerol backbone with the PKC–PS–$Ca^{2+}$ ternary complex. It appears that DO, the potent PKC activator, could permit an easy formation of such three-point attachment in the space-filling model on the surface of the phospholipid membrane, whereas DS, the poor activator, could not. Similarly, some unique physicochemical properties of diacylglycerols might be important factors governing their

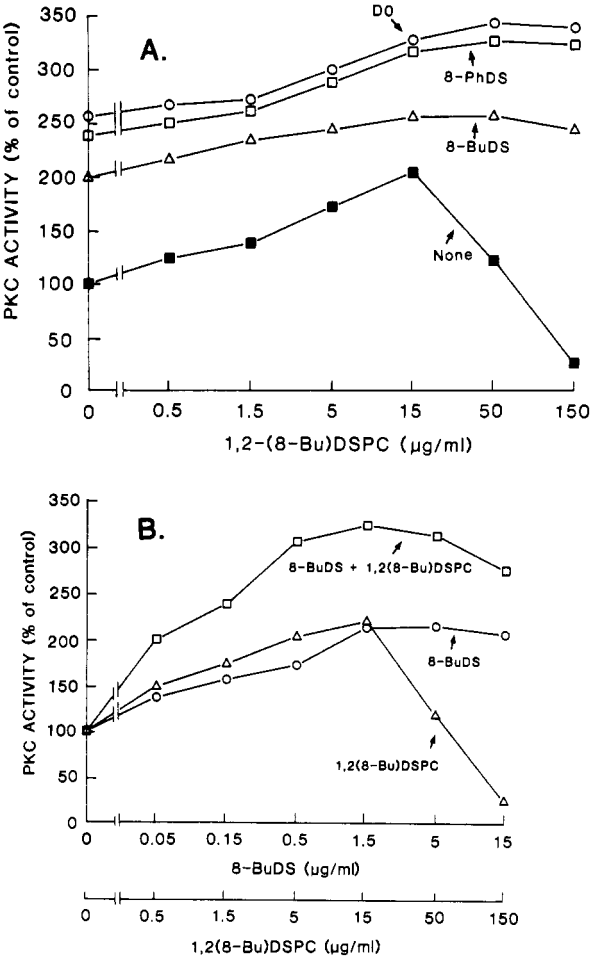



FIGURE 7: Effects of 1,2(8-Bu)DSPC and diacylglycerols, present singly or in combination, on PKC activity. (A) The enzyme was assayed in the presence of 10 μM $CaCl_2$, 10 μg/mL PS, 15 μg/mL DO, 8-PhDS, 8-BuDS, and/or varying concentrations of 1,2(8-Bu)DSPC, as indicated. (B) The enzyme was assayed in the presence of 10 μg/mL PS and varying concentrations of the DSPC and DS analogues, as indicated. The activity (16.2 pmol/min) seen in the absence of the analogues was taken as 100%. Similar results were obtained in two or three separate sets of experiments.

activity. It was observed in monolayer experiments that stearic acid chain distortion was induced by butyl or phenyl substitution but to a lesser extent by methyl substitution (F. M. Menger et al., unpublished results). This distortion (chain bending) might be similar to that seen for unsaturated fatty acids (i.e., arachidonic and oleic), both of which have been shown to be activators of PKC assayed in the presence or absence of PS and/or $Ca^{2+}$ (McPhail et al., 1984). It is likely, therefore, that octanoyl moieties in dioctanoylglycerol (Ganong et al., 1986), a potent activator, have a proper chain length and spatial orientation to permit an effective attachment without chain bending.

It is of interest that the similar effects of branched-chain substitutions were also observed previously with DSPC analogues (Charp et al., 1988b). Thus, methyl substitutions in DSPC, itself a weak PKC inhibitor, can potentiate its inhibitory activity, whereas butyl or phenyl substitutions can transform it to become a potent activator. One of the analogues, 1,2-(8-Bu)DSPC, unlike diacylglycerols (DO, 8-BuDS, or 8-PhDS), activates PKC without increasing its affinity for PS or $Ca^{2+}$. It, moreover, further stimulates PKC that is already activated by phorbol ester or diacylglycerol, without competing for [$^3$H]PDBu binding to PKC (Charp et al., 1988b). These findings, coupled with the additiveness of activation by 1,2(8-Bu)DSPC with that by DO, 8-BuDS, or

8-PhDS (Figure 7), strongly suggested that the PC and diacylglycerol analogues acted differently. We also observed recently that lyso-PC(oleoyl) activates PKC in the presence or absence of diacylglycerol, accompanied by an increased affinity for PS but a decreased affinity for $Ca^{2+}$ (Oishi et al., 1988). These findings indicate that lyso-PC has mechanisms of action that are likely distinct from those for diacylglycerols and DS and DSPC analogues, further suggesting a complexity of PKC regulation by lipids. Introduction of branched chains to phospholipid and/or diacylglycerol would represent a new approach with which to investigate molecular events and specificity crucial for PKC activation and inactivation.

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# Rat Kidney L-2-Hydroxyacid Oxidase. Structural and Mechanistic Comparison with Flavocytochrome $b_2$ from Baker's Yeast


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ABSTRACT: Hydroxyacid oxidase from rat kidney is an FMN-dependent enzyme that catalyzes the oxidation of L-$\alpha$-hydroxy acids as well as, more slowly, that of L-$\alpha$-amino acids. We report here a modified purification method for the enzyme, which is found to possess one cofactor per subunit of $M_r$ 39 000. Determination of its N-terminal sequence suggests the protein is homologous to spinach glycolate oxidase and baker's yeast lactate dehydrogenase. In the presence of a hydroxy acid and of bromopyruvate, under anaerobic conditions, the enzyme is found to catalyze both transhydrogenation and reductive bromide ion elimination. It had previously been observed that hydroxyacid oxidase could not catalyze chloride elimination from chlorolactate in the presence of oxygen [Cromartie, T. H., & Walsh, C. T. (1975) *Biochemistry 14*, 3482–3490]. The behavior of this enzyme toward halogeno substrates is therefore similar to that of baker's yeast L-lactate dehydrogenase and in part different from that of *Mycobacterium smegmatis* lactate oxidase and porcine kidney D-amino-acid oxidase. These findings can be rationalized on the basis of a common mechanism for all these enzymes, implying formation of a carbanion as a first step, with different rate-limiting steps in the overall reaction.


L-$\alpha$-Hydroxyacid oxidase from rat kidney (EC 1.1.3.15) is an FMN-containing flavoenzyme that oxidizes L-$\alpha$-hydroxy acids to keto acids with formation of hydrogen peroxide at the expense of oxygen (Blanchard et al., 1945, 1946). It was first isolated as an L-amino-acid oxidase (EC 1.4.3.2) (Blanchard et al., 1945) and subsequently shown to be more active with aliphatic longer chain homologues of glycolate, as well as with aromatic hydroxy acids such as mandelate and phenyllactate (Blanchard et al., 1946; Nakano & Danowski, 1966). More recently, Brush and Hamilton (1981) suggested that thiol–


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