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Evidence that a second stereochemical centre in diacylglycerols defines interaction at the recognition site on protein kinase C

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The interaction of novel diacylglycerol analogues at the recognition site on protein kinase C has been evaluated using a modified [^{3}H]phorbol dibutyrate binding assay and an established kinase activation assay. Studies with the 3-methyl analogues of 1,2-dihexanoyl-*sn*-glycerol have revealed a preferred stereochemical configuration at the C-3 position. Other chemical modifications have extended existing structure/activity relationships by showing that carbamates and sulphonyl esters cannot substitute for carboxylate esters and that cyclic acyl groups are active. Thus, most, if not all of the functionalities in the diacylglycerol molecule are required for interaction at the receptor on protein kinase C. Stereochemical specificity is required at C2 and C3.

Protein kinase C; Diacylglycerol; Phorbol ester; Structure/activity relationship

1. INTRODUCTION

Diacylglycerol (DAG) generated from phosphatidylinositol 4,5-bisphosphate hydrolysis within the plasma membrane activates protein kinase C (PKC) through an interaction that has characteristics of a ligand-receptor interaction [1–3]. Furthermore, the receptor on PKC for DAG is also the receptor for certain phorbol esters, such as phorbol 12-myristate-13-acetate (PMA) and phorbol 12,13-dibutyrate (PDBu). Understanding of the role of PKC in cellular control would be improved by refinement of the chemical tools with which to either stimulate or, more importantly, inhibit this enzyme.

To date only four publications have described in detail the structural requirements for activators of PKC. Ganong et al. [4], Boni and Rando [5] and Kerr and co-workers [6] focused on the structural requirements of DAGs for activation, while Wender et al. [7] used computer modelling techniques in an attempt to identify structural features common to PKC-activating natural products. Our aim has been to investigate further the structural features of the DAG molecule that confer affinity for the DAG binding site and efficacy for PKC activation.

This report extends current understanding of the structure/activity relationships for activation of PKC by DAGs and describes a second chiral centre in DAG-related molecules that defines further the stereochemical configuration necessary for interaction at the receptor on PKC.

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2. MATERIALS AND METHODS

PKC was partially purified by a procedure based on the method of Niedel et al. [8] and was assayed using the method described by Hannun et al. [9,10] which induced the following modifications: phosphatidylserine (PS) in the Triton X-100 mixed lipid micelles was increased to 20 mol%, histone IIIs was added at 1 mg/ml and the free calcium concentration was buffered at 100 μM [11].

[^{3}H]PDBu binding was measured using the PS/Triton X-100 mixed micelle system described previously [12]. A rapid filtering

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technique was developed in which PKC, its associated ligand and PS/Triton X-100 mixed micelles were bound to a beaded anion exchanger (DE52, Whatman) prior to filtration through a GF/C filter. DE52, supplied pre-swollen, was washed extensively in the assay buffer prior to use. The final concentration of [^{3}H]PDBu was 10 nM, PS was used at 20 mol% and non-specific binding was assessed in the presence of 10 μM unlabelled PDBu.

2.1. *Chemical synthesis*

(*R*)-4-Acetyl-2,2-dimethyl-1,3-dioxolane was prepared from methyl α,β-isopropylidene-L-glycerate (Fluka) by the method described [13] for the (*S*)-enantiomer. Reduction with lithium aluminium hydride gave a mixture of epimeric alcohols which on sequential benzylation [14], deketalisation [14] and dihexanoylation [15] produced a mixture of epimeric 3-benzyloxy-1,2-dihexanoyloxybutanes. These diesters were separated by flash chromatography, eluting with a mixture of ether and hexane (1:7). The faster running component was characterised and debenzylated [15] to give pure 2-(*R*)-hexanoyloxy-3-(*S*)-hydroxybutyl hexanoate (**I**, see fig.2). The slower running diester similarly gave the 2(*R*),3(*R*)-isomer (**II**, fig.2). The 2(*S*),3(*R*)- and 2(*S*),3(*S*)-isomers (**III** and **IV**, respectively, fig.2) were prepared exactly as described above starting with α,β-isopropylidene-D-glycerate. Other glycerol derivatives were prepared essentially by the methods described previously [15].

All products and intermediates were shown to be homogeneous by thin-layer chromatography, exhibited 200 MHz NMR spectra consistent with the proposed structures, and gave satisfactory microanalyses for carbon, hydrogen and, where appropriate, nitrogen.

2.2. *Materials*

PS was supplied by Lipid Products, England as the sodium salt. Leupeptin and PDBu were from Sigma and EGTA (Puriss grade) was obtained from Fluka. All other reagents were of analytical grade.

3. RESULTS

A series of novel diacylglycerols largely based on the structure of the cell-permeant compounds dihexanoylglycerol and dioctanoylglycerol [16–20], were synthesized and tested in the two assay systems. 1,2-Dihexanoyl-*sn*-glycerol had at least 10-fold greater affinity than its enantiomer, 2,3-dihexanoyl-*sn*-glycerol, or the positional isomer, 1,3-dihexanoylglycerol (fig.1A). The requirement for a 1,2-diacyl-*sn*-glycerol configuration was also evident from the PKC activation data, the enantiomer and the positional isomer being approx. 50 times less potent than 1,2-dihexanoyl-*sn*-glycerol (fig.1B). The stereochemical requirements for interaction at the recognition site were further explored by substituting methyl groups for the hydrogen atoms at the 3 position in 1,2-dihexanoyl-*sn*-glycerol. This chemical modification

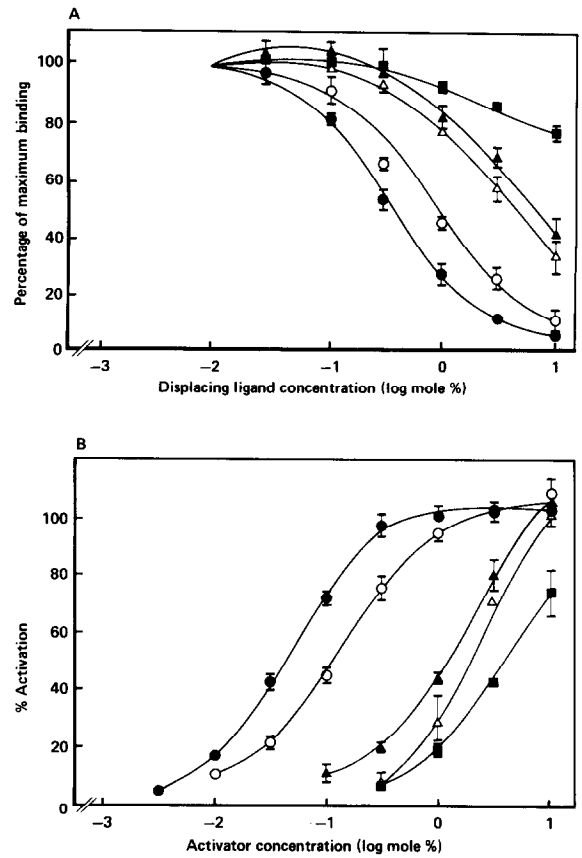

Fig.1. (A) Displacement of [^{3}H]PDBu from PKC by diacylglycerol analogues. (B) Activation of PKC by diacylglycerol analogues. Data are expressed as a percentage of the response stimulated by a saturating concentration of PDBu. PS and Ca^{2+}-dependent kinase activity was stimulated 335 ± 12% (mean ± SE of 10 determinations) by 10 μM PDBu. (●) 1,2-Dihexanoyl-*sn*-glycerol; (○) epimer **I**; (▲) 2,3-dihexanoyl-*sn*-glycerol; (△) 1,3-dihexanoylglycerol; (■) epimer **II**. All results are the mean ± SE of three separate determinations.

yields two epimers differing in configuration at the 3 position (fig.2, **I** and **II**). Epimer **I** had an ED_{50} for activation of PKC of 0.1 mol%, which is almost 50 times less than the ED_{50} for epimer **II** (see fig.1B). This difference in potency was reflected in the significant difference in the affinities of the epimers for the PDBu binding site (fig.1A). Epimer **I** displaced [^{3}H]PDBu with an IC_{50} value of 0.8 mol% whereas epimer **II** displaced only 25% of the total [^{3}H]PDBu at 10 mol%.

The set of methyl substituted diacyl compounds was completed by preparing the epimeric pair **III**

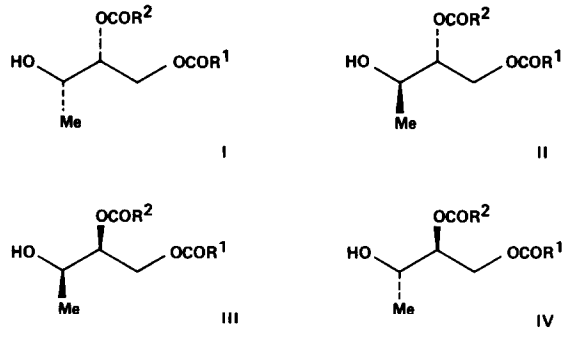

Fig.2. The 3-methyl isomers of dihexanoyl-*sn*-glycerol. **I**, 2-(*R*)-hexanoyloxy-3-(*S*)-hydroxybutyl hexanoate; **II**, 2-(*R*)-hexanoyloxy-3-(*R*)-hydroxybutyl hexanoate; **III**, 2-(*S*)-hexanoyloxy-3-(*R*)-hydroxybutyl hexanoate; **IV**, 2-(*S*)-hexanoyloxy-3-(*S*)-hydroxybutyl hexanoate.

and **IV** (see fig.2), which are mirror images of **I** and **II**, respectively, and have the unnatural stereochemistry at position 2. Epimers **III** and **IV** were very weak activators of PKC and had low affinity at the phorbol ester receptor displacing approx. 25% of the specifically bound [^{3}H]PDBu at 10 mol%.

A number of compounds with similar lipid solubility (calculated log *P* values) to 1,2-dihexanoyl-*sn*-glycerol were synthesized in order to probe the requirements for bulk and flexibility in the acyl chains independently from the demands for lipophilicity. Increasing the bulk and restricting the movement of the chains in cyclohexyl or cyclopentyl rings had no effect on either affinity or efficacy. Introduction of a phenyl group reduced the ability to activate PKC and slightly decreased affinity. However, this was largely overcome by including an extra methylene group between the ring and the glycerol backbone (not shown). It appears that the affinity and efficacy of these compounds is governed more by their lipophilicity than by the bulk and flexibility of the acyl chains. For example, compounds in which a methylene group in the acyl chain was replaced by an ether-linked oxygen did not displace [^{3}H]PDBu at concentrations up to 10 mol% and failed to activate PKC (not shown).

Replacing the carboxylate ester moieties at positions 1 and 2 with carbamoyl groups or sulphonyl esters caused a significant drop in affinity and efficacy. Substituting only one of the carboxylate ester groups at either position 1 or 2 with a carbamoyl or sulphonyl moiety also resulted in a dramatic loss of potency in both assay systems (not shown). Eliminating the hydroxyl group at position 3 yielded an inactive compound which failed to displace [^{3}H]PDBu or activate PKC even at high concentrations (not shown). Likewise, substituting an aldehyde function at this position also produced a compound that had no activity in the two assay systems.

4. DISCUSSION

This report forms part of a continuing investigation of the structural requirements for interaction at the DAG site on PKC. Inherent in this study, as in others, is the assumption that the relevant recognition sites behave the same in detergent/lipid micelles as in the phospholipid bilayer of plasma membranes. The histone phosphorylation assay was characterised in detail to obtain optimum conditions for measuring DAG-stimulated kinase activity. The optimum concentration of PS was 20 mol%, rather than 10 mol% as reported by Hannun et al. [10,12]: in our hands PS at 10 mol% supported only a very small degree of kinase activity. Nevertheless, the rank order of potency of the short-chain DAGs agrees exactly with published reports [18,20] although the concentration-effect curves for activation of PKC are to the left of those reported previously [10]. The structural requirements for PKC activators derived from isolated enzyme studies [4,5], have been confirmed by investigations using whole cells [18–24]. Clearly the interaction of DAGs with PKC in cell membranes can be reproduced in the PS/Triton mixed micelle assay.

Analysis of the [^{3}H]PDBu binding assay used in this study indicated that association of the enzyme with DEAE cellulose did not affect the binding parameters, the apparent K_d (12 nM) and the time course of [^{3}H]PDBu binding (not shown) being very close to those observed previously [12]. Furthermore, the displacement of [^{3}H]PDBu by DAG displayed the stereospecificity seen in the histone phosphorylation assay (fig.1A and B and [5,23]) and in DAG stimulation of whole cells [21,22]. The 10-fold greater affinity of the 1,2-diacyl-*sn*-enantiomer is exactly that observed in whole HL60 cells (Thompson, N.T., unpublished).

The novel structural modifications made to the

DAG molecule described here have provided further insight into the requirements for affinity, but at present these are indistinguishable from the requirements for efficacy. Analysis of the two pairs of diastereoisomers (**I–IV**, fig.2) has extended our understanding of the stereochemical requirements for interaction with the recognition site on PKC. In addition to the need for a natural 1,2-diacyl-*sn*-glycerol configuration [5,21–23] there is a preferred stereochemical arrangement at the 3 position in the methylated analogues. Future analysis of the 1-methyl isomers will help further to define the absolute stereochemical configuration of DAG-like PKC activators. This study also confirms and extends a number of the structural features required for interaction at the DAG receptor on PKC. Carbamates and sulphonyl esters cannot substitute for carboxylate esters, furthermore, alkyl substitutions at either C1 or C2 also resulted in a loss of activity, supporting the conclusion that both carbonyl functions are necessary for full potency [4]. There is an absolute requirement for a 3-hydroxyl moiety [4]. Replacing the hydroxyl group with an aldehyde function does not restore either affinity or efficacy, suggesting that the primary hydroxyl group is hydrogen-bond donating. There is clearly a strict requirement for lipophilicity in the acyl chain; a compound which contains an oxygen atom in the middle of the chain is significantly less lipophilic than 1,2-dihexanoyl-*sn*-glycerol and was devoid of activity. We have no evidence to suggest that this lack of activity was due to the compound being unable to form mixed lipid micelles. Using the PS/Triton mixed micelle assay Hannun et al. [10] showed that DAGs with acyl chains containing 8 or more carbon atoms were the most potent activators of PKC. Our investigation extends this observation by showing that both affinity and efficacy are relatively independent of the bulk and electronic properties of the acyl chains.

Thus, it is apparent that effective interaction between DAG and the recognition site on PKC requires all of the chemical functionalities of the DAG molecule and a more precise stereochemical configuration than has been described before.

REFERENCES

[1] Hirasawa, K. and Nishizuka, Y. (1985) Annu. Rev. Pharmacol. Toxicol. 25, 147–170.

[2] Berridge, M. (1986) Biol. Chem. Hoppe-Seyler 367, 447–456.

[3] Taylor, C.W. and Merritt, J.E. (1986) Trends Pharmacol. Sci. 7, 238–242.

[4] Ganong, B.R., Loomis, C.R., Hannun, Y.A. and Bell, R.M. (1986) Proc. Natl. Acad. Sci. USA 83, 1184–1188.

[5] Boni, L.T. and Rando, R.R. (1985) J. Biol. Chem. 260, 10819–10825.

[6] Kerr, D.E., Kissinger, L.F., Gentry, L.E., Purchio, A.F. and Shoyab, M. (1987) Biochem. Biophys. Res. Commun. 148, 776–782.

[7] Wender, P.A., Koehler, K.F., Sharkey, N.A., Dell'Aquila, M.L. and Blumberg, P.M. (1986) Proc. Natl. Acad. Sci. USA 83, 4214–4218.

[8] Niedel, J.E., Kuhn, L.J. and Vandenbark, G.R. (1983) Proc. Natl. Acad. Sci. USA 80, 36–40.

[9] Hannun, Y.A., Loomis, C.R. and Bell, R.M. (1985) J. Biol. Chem. 260, 10039–10043.

[10] Hannun, Y.A., Loomis, C.R. and Bell, R.M. (1986) J. Biol. Chem. 261, 7184–7190.

[11] Fabiato, A. and Fabiato, J. (1979) J. Physiol. (Paris) 75, 463–505.

[12] Hannun, Y.A. and Bell, R.M. (1986) J. Biol. Chem. 261, 9341–9347.

[13] Tanner, D. and Somfai, P. (1986) Synth. Commun. 16, 1517–1522.

[14] Mulzer, J. and Angermann, A. (1983) Tetrahedron Lett. 24, 2843–2846.

[15] Dawson, J., Thompson, N.T., Bonser, R.W., Hodson, H.F. and Garland, L.G. (1987) FEBS Lett. 214, 171–175.

[16] Lapetina, E.G., Reep, B., Ganong, B.R. and Bell, R.M. (1985) J. Biol. Chem. 260, 1358–1361.

[17] Davis, R.J., Ganong, B.R., Bell, R.M. and Czech, M.P. (1985) J. Biol. Chem. 260, 1562–1566.

[18] Ebeling, J.G., Vandenbark, G.R., Kuhn, L.J., Ganong, B.R., Bell, R.M. and Niedel, J.E. (1985) Proc. Natl. Acad. Sci. USA 82, 815–819.

[19] Jetten, A.M., Ganong, B.R., Vandenbark, G.R., Shirley, J.E. and Bell, R.M. (1985) Proc. Natl. Acad. Sci. USA 82, 1941–1945.

[20] Davis, R.J., Ganong, B.R., Bell, R.M. and Czech, M.P. (1985) J. Biol. Chem. 260, 5315–5322.

[21] Nomura, H., Ase, K., Sekiguchi, K., Kikkawa, U., Nishizuka, Y., Nakano, Y. and Satoh, T. (1986) Biochem. Biophys. Res. Commun. 140, 1143–1151.

[22] Bonser, R.W., Dawson, J., Thompson, N.T., Hodson, H.F. and Garland, L.G. (1986) FEBS Lett. 209, 134–138.

[23] Rando, R.R. and Young, N. (1984) Biochem. Biophys. Res. Commun. 122, 818–823.

[24] Conn, P.M., Ganong, B.R., Ebling, J., Staley, D., Niedel, J.E. and Bell, R.M. (1985) Biochem. Biophys. Res. Commun. 126, 532–539.