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Alkyl analogs of diacylglycerol as activators of protein kinase C

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Diacylglycerols which activate protein kinase C have the 1,2-*sn* configuration. Short-chain saturated fatty acids or long-chain unsaturated fatty acids are required for supporting the potency of these lipids. Using alkyl analogs such as 1-*O*-decyl-2-*O*-decanoylglycerol, 1-*O*-decanoyl-2-*O*-decylglycerol, 1,2-*O*-didecylglycerol, 1-*O*-hexadecyl-2-*O*-acetylglycerol and 1-*O*-decyl-2-*O*-acetylglycerol, we showed that the ether bond was consistently associated with a loss of activity to varying extents. The results suggest that the ester bond in the 1-position is a major determinant of diacylglycerol-mediated protein kinase activation.

Protein kinase C; Diacylglycerol; Alkyl analog

1. INTRODUCTION

Extracellular ligands produce signals from the surface which are transduced into the cell through various strategies. Signaling of a large class of ligands, including hormones, neurotransmitters and mitogens, appears to be mediated through Ca^{2+} mobilization and protein kinase C activation [1,2]. Upon interaction with specific receptors, the ligand enhances the polyphosphoinositide turnover which results in the transient accumulation of diacylglycerol and inositol 1,4,5-trisphosphate. The neutral lipid is the physiological activator of the protein kinase C, while inositol 1,4,5-trisphosphate mobilizes Ca^{2+} from its internal stores. As a consequence, this class of ligands control both Ca^{2+}-dependent processes and protein kinase C-catalyzed protein phosphorylation (reviews [3-5]). Tumor-promoting phorbol esters substitute for diacylglycerol in activating protein kinase C [6]. However, the involvement of this enzyme in tumor promotion remains to be assessed.

Some specific structural requirements of diacylglycerol as well as phorbol esters for protein kinase C activation have been shown in early reports [6,7]. Thereafter, in biochemical and theoretical studies, we have shown that the hydroxyl group in the 3-position of diacylglycerol was required for activity, and we have emphasized the importance of its orientation into the lipid bilayer [8,9]. In addition, protein kinase C activation has been described as stereospecific [10,11] and therefore 1,2-*sn* appeared to be the active configuration of diacylglycerol. Moreover, it has been reported that esterification with saturated short-chain fatty acid or unsaturated long-chain fatty acid led to greater efficiency of these compounds [8,12,13].

We have carried out further experiments in order to evaluate the importance of ester bonds in the activation process of protein kinase C, both in

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Abbreviations: diC_{10}, 1,2-*O*-didecanoyl-*rac*-glycerol; AAG-16, 1-*O*-hexadecyl-2-*O*-acetylglycerol; AAG-18, 1-*O*-octadecyl-2-*O*-acetylglycerol; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; DMF, dimethylformamide; TMS, tetramethylsilane

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vitro and in intact cells. We report here that the introduction of an ether bond in the l-position caused a dramatic loss of activity whereas, at variance, an ether bond in the 2-position yielded a compound which was still active, though markedly less so than the diacylglycerol counterpart.

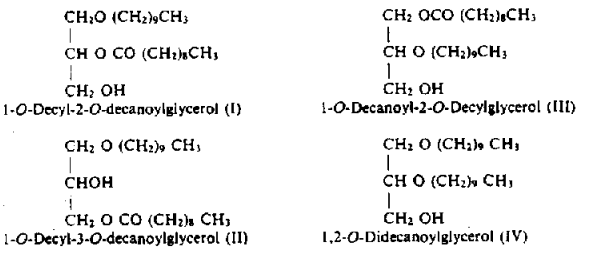
2. MATERIALS AND METHODS

2.1. *Materials and chemicals*

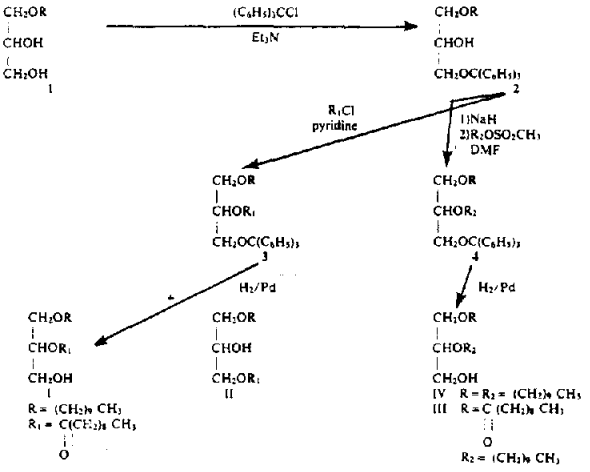
Rabbit platelets were washed according to Baenziger and Majerus [14]. Protein kinase C was partially purified from mouse brain as described [15]. [γ-^{32}P]ATP was obtained from New England Nuclear (USA) and carrier-free ^{32}P from Amersham (England). TPA was purchased from CCR (USA). R 59022 was kindly provided by Dr de Chaffoy de Courcelles (Janssen Pharmaceutica Research Laboratories); 1-*O*-octadecyl-2-acetyl-*sn*-glycerol was provided by Novabiochem and other chemicals including 1,2-*rac*-didecanoylglycerol were obtained from Sigma (USA).

2.2. *Synthetic procedure*

As racemic mixtures, the following compounds were synthesized:



Alkylacyl and dialkylglycerols were prepared mainly as in [16,17], including several modifications. 1-*O*-Decylglycerol (**1**) prepared according to Baumann et al. [18] was converted into its trityl derivative, **2** (yield 75%), and esterified in **3** by classical action of decanoyl chloride in benzene/pyridine (yield 91%). Catalytic hydrogenolysis (10% Pd/C) of **3** led to a mixture of the two positional isomers I and II due to spontaneous acyl migration (yield 85%). Separation of I and II was performed by chromatography on a silica gel column using 15% ether in petroleum ether as eluent. Etherification of **2** by prior treatment with sodium hydride (60°C, 30 min) followed by treatment with decylmethanesulfonate (80°C, 2 H) in DMF led to the triether **4** (yield 64%) which was hydrogenolysed in the same manner as **3** to obtain 1-*O*-2-*O*-didecylglycerol, IV (yield 76%). Preparation of 1-*O*-decanoyl-2-*O*-decylglycerol, (III) was performed according the same procedure as for IV starting from 1-*O*-decanoylglycerol. The whole procedure is summarized below.



All structures were confirmed by IR (Pye-Unicam SP_3-200) and ^{1}H NMR (Varian EM 360 or Brücker 250 MHz) in $CDCl_3$ with TMS as an internal standard. Elemental analyses were consistent with the proposed structures. The physicochemical properties and spectral data of the final compounds are summarized in table 1.

In addition, 1-*O*-octadecyl-2-*O*-acetyl-*rac*-glycerol was synthesized using the procedure described above.

2.3. *Assay for protein kinase C in cell-free system*

Protein kinase C was assayed as in [2]. The reaction mixture (125 μl) contained 2.5 μmol Tris-HCl buffer at pH 7.5, 10 μg phosphatidylserine or brain extract type III from Sigma, 0.5 μmol magnesium acetate, 2.5 nmol [γ-^{32}P]ATP (approx. 3×10^5 dpm), 15 μg histone H_1 and 0.4–0.5 μg enzyme preparation. Diacylglycerol and analogs, $CaCl_2$ and EGTA were added as indicated in the text. Neutral lipids and phospholipids were dissolved in chloroform, then dried under an N_2 stream and resuspended in the incubation buffer by sonication

Table 1

Physicochemical constants of alkylacylglycerols I and II, acylglycerol III and dialkylglycerol IV

Compound	Formula	IR[a](KBr)ν(cm^{-1})	^1H NMR [a](MHz) (ppm)
I[b]	$CH_2O(CH_2)_9CH_3$ | $CHOCO\ (CH_2)_8CH_3$ | CH_2OH	3460 (O-H), 1740 (C=O), 1170, 1120 (C-O-C)	250 MHz: 4.99 (quintet, 1H, CHOCO), 3.83 (m,2H, CH_2OH), 3.64 (m,2H, CH_2OR), 3.47 (m,2H, OCH_2), 2.37 (t,2H, $OCOCH_2$)
II[b]	$CH_2O(CH_2)_9CH_3$ | $CHOH$ | $CH_2OCO(CH_2)_8CH_3$	3400 (O-H), 1740 (C=O), 1175, 1125 (C-O-C)	250 MHz:4.14 (m,2H, CH_2OCO), 3.46 (m,3H, $2CH_2O+CHO$), 2.35 (t,2H, $OCOCH_2$)
III[b]	$CH_2O(CH_2)_9CH_3$ | $CHO(CH_2)_9CH_3$ | CH_2OH	3460 (O-H), 1130 (C-O-C)	80 MHz:3.57 (m,9H, $4CH_2O+CHO$)
IV[b]	$CH_2OCO(CH_2)_8CH_3$ | $CHO(CH_2)_9CH_3$ | CH_2OH	3450 (O-H), 1740 (C=O), 1090 (C-O)	250 MHz:4.15 (m,2H, CH_2OCO), 3.51 (m,5H, $2CH_2O+CHO$), 2.32 (quadruplet, 2H, $OCOCH_2$)

[a] Key peaks
[b] Viscous oil at room temperature

as described [19]. Incubation was carried out for 3 min at 30°C. The reaction was stopped by transferring 50 μl aliquots onto squares of Whatman paper ET31 followed by several 25% trichloroacetic acid baths. The radioactivity was determined by Cerenkov radiations.

2.4. *Protein kinase C assay in intact platelets*

Washed cells (4×10^9) were labeled with 0.5 mCi $^{32}P_i$ in 1 ml phosphate-free Tyrode's solution containing 1 mM EGTA, 0.25% BSA and 10 mM Tris-HCl buffer (pH 7.4), at room temperature. The platelets were then spun down and resuspended in the same buffer (50 μl) containing 1.5 mM $CaCl_2$ instead of 1 mM EGTA. Stimulation with thrombin, diacylglycerol and analogs was as indicated in the figure legends. Neutral lipids were added as sonicates in the incubation buffer. Labeled proteins were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The relative intensity of the 43 kDa protein was quantified by densitometric tracing of the autoradiogram using a Joyce and Loebl chromatogram scanner.

3. RESULTS AND DISCUSSION

1,2-*O*-Dioleoylglycerol and 1,2-*O*-didecanoylglycerol (diC_{10}) are currently used as exogenous activators. Although the two compounds exhibit a similar potency in the in vitro assay, the latter more easily permeates intact cells. With the purpose of examining the importance of the ester bond, we prepared analogs of diC_{10} containing one or two ether bonds. In addition, we tested the activity of two precursors of the platelet-activating factor: 1-*O*-hexadecyl-2-*O*-acetylglycerol (AAG-16) and 1-*O*-octadecyl-2-*O*-acetylglycerol (AAG-18).

In vitro tests using mouse brain enzyme have shown that the monoether in the 2-position (III), though stimulating the enzyme at concentrations higher than diC_{10}, elicited a marked activating ef-

fect. In contrast, monoether in the 1-position (I) and diether IV were poorly active in this assay. Likewise, 1-alkyl-3-acylglycerol II was revealed as being devoid of activity (fig.1). It should be noted that the experiments were performed in the presence of either 10 μM $CaCl_2$ or 0.5 mM EGTA and that results were essentially similar.

Further tests were conducted in intact platelets. One of the major substrates of protein kinase C in platelets is a protein of 43 kDa. The degree of phosphorylation of this substrate was used as an index of the enzyme activity in these cells. Assuming that alkyl analogs are as permeable as the diacylglycerol parent, the replacement of ester bonds by ether bonds leads to a marked loss of activity which affects, like in the in vitro assay, preferentially the monoether in the 1-position, and the diether. The activity of the latter is approximately one-tenth that of diC_{10}, as shown in fig.2.

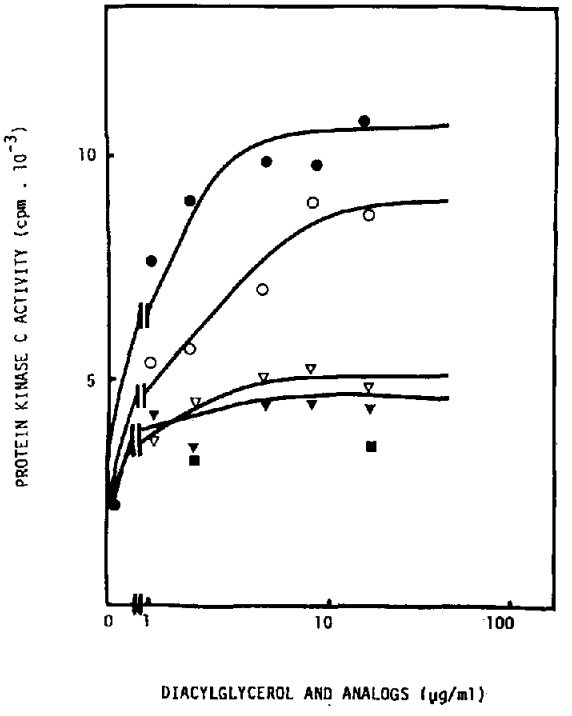


Fig.1. Dose dependence of protein kinase C activation in response to diC_{10} (●) and analogs: 1-*O*-decyl-2-*O*-decanoyl-*rac*-glycerol (▽); 1-*O*-decanoyl-2-*O*-decyl-*rac*-glycerol (○) 1,2-*O*-didecyl-*rac*-glycerol (▽) and 1-*O*-decyl-3-*O*-decanoyl-*rac*-glycerol (■). The assay was performed as described in section 2; 0.5 mM EGTA was added. The experiment shown is representative of 3 superimposable experiments.

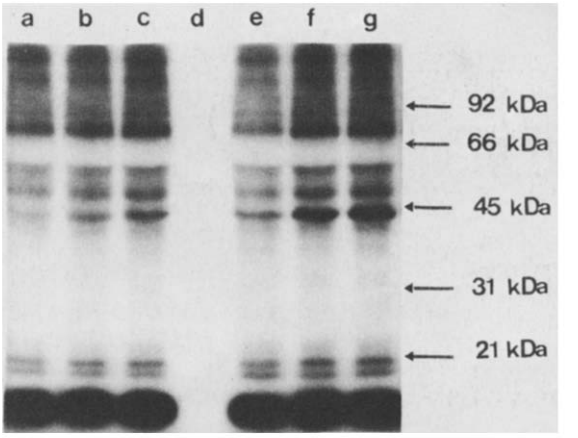


Fig.2. Effect of diacylglycerol diC_{10} and diether analog on phosphorylation of 43 kDa protein. Cells were incubated at 37°C in Tyrode's solution (lane a) or 1,2-*O*-didecyl-*rac*-glycerol at 20 μg/ml (lane b) and 100 μg/ml (lane c) or 1,2-*O*-didecyl-*rac*-glycerol at 2 μg/ml (lane 3), 20 μg/ml (lane f) and 100 μg/ml (lane g). The molecular mass markers were run in lane d.

As a result of time course experiments using the monoether in C-2 (III), we were able to show that replacement of the 2-ester did not affect the half-life of the phosphorylated state of the protein 43 kDa which was close to 30 min (fig.3). The addi-

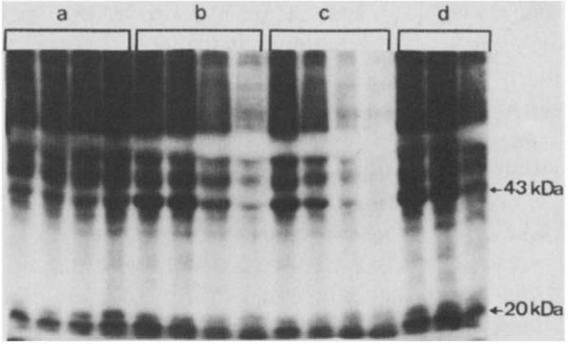


Fig.3. Autoradiogram of SDS-polyacrylamide gel electrophoresis of platelet proteins. Cells were washed and ^{32}P labeled as described in section 2, then incubated at 37°C in Tyrode's solution (lanes a) containing either 100 μg/ml of 1-*O*-decyl-2-*O*-decanoyl-*rac*-glycerol (lanes b), 100 μg/ml of 1-*O*-decyl-2-*O*-decanoyl-*rac*-glycerol + 0.1 mM R 59022 (lanes c) or 100 μg/ml of diC_{10} (lanes d) for 1 min, 15 min, 30 min and 1 h (from left to right lanes) except for diC_{10} treatment, which was for only 1, 15 and 30 min.

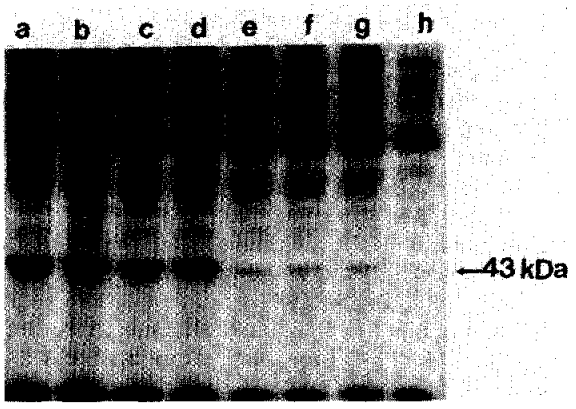


Fig.4. Autoradiogram of SDS-polyacrylamide gel electrophoresis: Time course of 1-*O*-hexadecyl-2-*O*-acetylglycerol-induced phosphorylation of 43 kDa protein in platelets. Cells were stimulated at 37°C with either 50 ng/ml TPA for 1 min (lane a) and 2 min (lane b) with 2 units/ml thrombin for 30 s (lane c) and 1 min (lane d) or with 120 μg/ml 1-*O*-hexadecyl-2-*O*-acetylglycerol for 30 s (lane e), 1 min (lane f) and 2 min (lane g). Unstimulated controls (lane h).

tion of R 59022, an inhibitor of diacylglycerol kinase [20], no longer sustained the phosphorylated state. The rapid drop in degree of phosphorylation suggests that diacylglycerol (and analogs)-induced platelet activation may be associated with a concomitant increase in protein phosphatase activity. Alternatively, dephosphorylation may account for a possible activator-induced 'down regulation' of protein kinase C in these cells [21]. Conversely, the 43 kDa protein may have become more intensively phosphorylated as a function of time in control samples, presumably due to slowly occurring spontaneous platelet activation.

Furthermore, we investigated the effects of 1-*O*-hexadecyl-2-*O*-acetylglycerol (AAG-16) from commercial sources and 1-*O*-octadecyl-2-*O*-acetylglycerol (AAG-18) synthesized in the laboratory. These two precursors of the platelet-activating factor are poorly active in the in vitro assay (not shown). However, AAG-16 exhibited a small but detectable activity in platelets whereas AAG-18 was totally inactive. The effects of usual activators, thrombin and TPA, on protein phosphorylation were compared with those of AAG-16, as shown in fig.4. It has been reported that AAG-16 induces, like TPA, the differentiation of promyelocytic cells HL60 to macrophage-like cells [22]. Our results suggest that the biological activity of this compound may actually result from protein kinase C activation.

In summary, the substitution of the ether for the ester bond in the diacylglycerol molecule leads to a loss of activity which is more drastic when the alkyl group is in the 1-position. These results are in agreement with recent studies conducted in a mixed micelle assay by Ganong et al. [23], which have shown that an amide linkage in C-2, and not in C-1, yields an active analog of diacylglycerol. It is of interest to note that the ether in C-2 has the potential for being a good tool in investigating the physiological role of protein kinase C.

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REFERENCES

[1] Nishizuka, Y. (1984) Science 225, 1365–1370.
[2] Berridge, M.J. and Irvine, R.F. (1984) Nature 312, 315–321.
[3] Berridge, M.J. (1984) Biochem. J. 220, 345–360.
[4] Nishizuka, Y. (1986) Science 233, 305–312.
[5] Castagna, M. (1987) Biol. Cell 59, 3–14.
[6] Castagna, M., Takai, Y., kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7874–7851.
[7] Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U. and Nishizuka, Y. (1980) J. Biol. Chem. 255, 2273–2276.
[8] Couturier, A., Bazgar, S. and Castagna, M. (1984) Biochem. Biophys. Res. Commun. 121, 448–455.
[9] Brasseur, R., Cabiaux, V., Huart, P., Castagna, M., Bazgar, S. and Ruysschaert, J.M. (1985) Biochem. Biophys. Res. Commun. 127, 969–976.
[10] Rando, R.R. and Young, N. (1984) Biochem. Biophys. Res. Commun. 122, 818–823.
[11] Nomura, H., Ase, K., Sekiguchi, K., Kikkawa, U. and Nishizuka, Y. (1986) Biochem. Biophys. Res. Commun. 140, 1143–1151.
[12] Lapetina, E.G., Reep, B., Ganong, and Bell, R.M. (1985) J. Biol. Chem. 260, 1358–1361.
[13] Davis, R.J., Ganong, B.R., Bell, R.M. and Czech, M.P. (1985) J. Biol. Chem. 260, 1562–1566.

[14] Baenziger, H.L. and Majerus, P.W. (1974) Methods Enzymol. 31, 149–155.
[15] Roghani, M., Da Silva, C. and Castagna, M. (1987) Biochem. Biophys. Res. Commun. 142, 738–744.
[16] Godfroid, J.J., Heymans, F., Michel, E., Redeuilh, C., Steiner, E. and Benveniste, J. (1980) FEBS Lett. 116, 161–164.
[17] Heymans, F., Michel, E., Borrel, M.C., Wichrowiski, B., Godfroid, J.J., Convert, O., Coeffier, E., Tence, M. and Benveniste, J. (1981) Biochim. Biophys. Acta 666, 230–237.
[18] Baumann, W.J. and Mangold, H.K. (1964) J. Org. Chem. 29, 3055–3057.
[19] Takai, Y., Kishimoto, A., Kikkawa, U., Mori, T. and Nishizuka, Y. (1979) J. Biol. Chem. 254, 3692–3695.
[20] De Chaffoy de Courcelles, D., Roevens, P. and Van Belle, H. (1985) J. Biol. Chem. 260, 15762–15770.
[21] Rodriguez, P. and Rozengurt, E. (1984) Biochem. Biophys. Res. Commun. 120, 1053–1059.
[22] McNamara, M.J.C., Schmitt, J.D., Wykle, R.L. and Daniel, L.W. (1984) Biochem. Biophys. Res. Commun. 122, 824–830.
[23] Ganong, B.R., Loomis, C.R., Hannun, Y.A. and Bell, R.M. (1986) Proc. Natl. Acad. Sci. USA 83, 1184–1188.