Vol. 151, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 29, 1988 Pages 291-297

ALKYL-LINKED DIGLYCERIDES INHIBIT PROTEIN KINASE C ACTIVATION BY DIACYLGLYCEROLS

Larry W. Daniel,* George W. Small, and Jeffrey D. Schmitt

Department of Biochemistry, Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, NC 27103

Canio J. Marasco, Khalid Ishaq, and Claude Piantadosi

Division of Medicinal Chemistry and Natural Products, School of Pharmacy, University of North Carolina, Chapel Hill, NC 27599-7360

Received January 20, 1988

Alkylacylglycerols are synthesized when choline-phospholipids are degraded by a phospholipase C. This class of compounds has been shown to have biological activities; however, the mechanism of action is unknown. A series of alkyl-linked diglycerides were synthesized and tested for activity in an in vitro assay for protein kinase C. When protein kinase C activity was stimulated with the synthetic diacylglyceride analog 1-oleoyl-2-acetyl-sn-glycerol, the addition of alkyl glycerides caused a concentration-dependent inhibition of protein kinase C activity. Comparison of the protein kinase C inhibition by this series of 1-O-alkyl-2-acyl analogs revealed that both saturated and unsaturated long-chain groups in position 1 were effective and that dietherglycerols with short-chain moities in position 2 were also effective. It is concluded from these studies that the biological activity of alkyl-linked glycerides may be expressed through protein kinase C inhibition.

Diacylglycerol produced by the receptor-mediated hydrolysis of inositol phospholipids acts as an intracellular messenger to bind to and activate protein kinase C (PKC) (1). We have shown that alkylacylglycerol is also formed after cells are stimulated by TPA (2). The source of this class of diglycerides is PC rather than the inositol phospholipids. Others have observed PC hydrolysis by a phospholipase C in a variety of cell types (3-8) and this appears to be an alternative pathway for the generation of diglyceride

*To whom correspondence should be addressed.

Abbreviations used: PKC, protein kinase C; TPA, 12-O-tetradecanoyl-phorbol-13-acetate; PC, 1-radyl-2-acyl-sn-glycero-3-phosphocholine; AAG(16:0), 1-O-hexadecy 2-acetyl-sn-glycerol; AAG(18:1), 1-O-Δ^9-octadecenyl-2-acetyl-sn-glycerol; OAG, 1-oleoyl-2-acetyl-sn-glycerol; AMG, 1-O-hexadecyl-2-O-methyl-rac-glycerol; AEG, 1-O-hexadecyl-2-O-ethyl-rac-glycerol; ET-18-OCH_3, 1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine.

0006-291X/88 $1.50

second-messengers (2,3). The alkylglycerides, in contrast to the diacylglycerides, are not effective stimulators of PKC (9,10). However, a synthetic alkylglyceride AAG(16:0), unlike the diacylglycerol OAG, was found to inhibit HL-60 cell growth and stimulate differentiation to macrophage-like cells (11). Therefore, the alkylglycerides have biological activities although they do not stimulate PKC. The diether phospholipid ET-18-OCH_3 also inhibits the growth of HL-60 cells (12) and stimulates differentiation (13). We have shown that the major metabolite of ET-18-OCH_3 in HL-60 cells is the corresponding dialkylglyceride (14) which may have biological activity similar to the alkylacylglycerides.

Because of the interesting biological properties of these alkylglycerides, we have synthesized a number of structural analogs of the alkyl- and dialkylglycerides and tested their activity in an in vitro PKC assay system. Our results indicate that the alkyl analogs inhibit diacylglycerol-stimulated PKC activity.

Experimental Procedures

Materials: DEAE-Sephacel was from Pharmacia P-L Biochemicals. Histone type IIIS, bovine serum albumin, β-mercaptoethanol and phenylmethylsulfonyl fluoride were from Sigma. [γ-^{32}P]ATP (8-12 Ci/mmol) was from New England Nuclear Research Products. Fetal bovine serum was from Gibco Laboratories, whereas medium and antibiotics for tissue culture were from Flow Laboratories, Rockville, MD. OAG and AAG(16:0) were prepared and their purity was determined as previously described (11). AAG(18:1) was prepared by the procedure described for AAG(16:0) except that selachyl alcohol (1-O-9'-octadecenyl-sn-glycerol) was used as the starting material. AMG and AEG were prepared by a method similar to that described previously (15). 1-O-Hexadecyl-rac-glycerol was protected at the primary alcohol by conversion to the trityl ether derivative as described by Muramatsu (16). The secondary alcohol was then alkylated with methyl or ethyl iodide and sodium hydride as described by Morris-Natschke et al. (15). The resulting products were then converted to AMG and AEG by removing the trityl protecting group with p-toluenesulfonic acid as described by Wissner et al. (17). The products were then purified and the expected products were confirmed by NMR spectroscopy as previously described (15).

Preparation of Protein Kinase C: PKC was prepared and assayed as described previously (18) with minor modifications as specified below. HL-60 cells were grown in 75 mL flasks, harvested and washed with ice-cold normal saline. After centrifugation (600 x g, 5 min, 4°C), the cell pellet was resuspended (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 10 mM EGTA, 50 mM β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride) and sonicated for 20 s with a stepped microprobe sonicator. Unbroken cells were removed by centrifugation as above and the supernatant centrifuged (65,000 x g, 90 min, 4°C). The supernatant from this step (cytosol) was then fractionated on a 1 x 8 cm DEAE-Sephacel column after

addition of sucrose to a final concentration of 10%. After equilibrating the column (20 mM Tris, pH 7.5, 0.2 mM EDTA, 0.2 mM EGTA, 50 mM β-mercaptoethanol, 10% sucrose), the sample was loaded and unbound material was washed through with 40 mL of the equilibration buffer. Then PKC was eluted by a gradient from 0-0.5 M NaCl in the buffer described above. Fractions of 1 mL were collected at 25 mL/h and 0.05 mL aliquots assayed for PKC activity as described below. The fractions with the highest activity were pooled and used in further experiments.

Assay of Protein Kinase C Activity: The assays were done at pH 7.5 in a total volume of 0.25 mL and all tubes contained 25 mM Tris, 10 mM $MgCl_2$, 40 μg/mL histone, 10 μM ATP (including 1 μCi of [γ-^{32}P]ATP), 0.1 μM $CaCl_2$, 20 μg/mL phosphatidylserine, 0.05 mL of the PKC preparation and the indicated concentrations of OAG. Enzymatic activity was determined as the incorporation of ^{32}P from [γ-^{32}P]ATP into histone in the presence of Ca^{2+}, phosphatidylserine, and the indicated concentration of OAG. Reactions were initiated by the addition of the enzyme preparation and halted after 20 min at 30°C by the addition of 0.05 mL bovine serum albumin (10 mg/mL) and 1 mL 25% ice-cold trichloroacetic acid. The tubes were kept on ice and then filtered in a Millipore vacuum box using Millipore HA filters and washed with 25% trichloroacetic acid. The radioactivity bound to the filters was determined by scintillation counting in 5 mL Budget Solve. The amount of enzyme used was shown to result in linear activity for at least 20 min and the assay was linearly dependent on the amount of enzyme used. The analog to be tested was added directly to the reaction mixture before the addition of PKC. As a control 0.1% ethanol was included in the samples with the enzyme but with no inhibitor.

Results

To characterize the effect of diacylglycerol, we assayed the PKC preparation from HL-60 cells with the synthetic diacylglycerol OAG. As shown in Figure 1, OAG caused a concentration-dependent activation of PKC with half-maximal activation by <2 μM OAG. However, the alkyl-linked analog of OAG, AAG (18:1), did not cause a significant stimulation of PKC activity (Fig. 1).

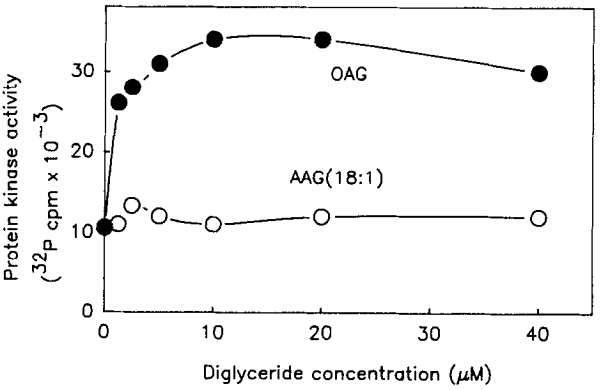

Fig. 1. Stimulation of protein kinase activity by diglyceride analogs. Total protein kinase activity was measured using a partially purified preparation from HL-60 cells. Total activity is defined as ^{32}P cpm incorporated into histone in the assay as described in "Experimental Procedures". Data are average of duplicates from a representative experiment. OAG, •—•; AAG (18:1), o—o.

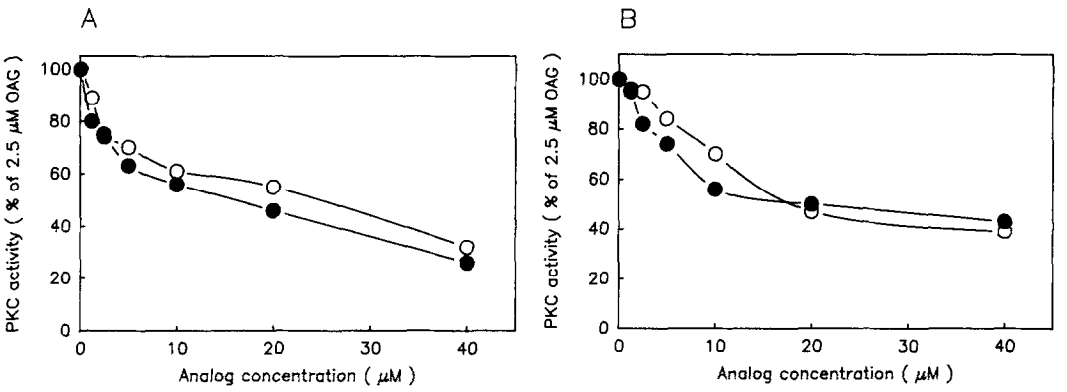

Fig. 2. Effect of alkylglycerides on OAG-stimulated PKC activity. PKC activity was measured in the presence of OAG, 2.5 μM, and the indicated concentration of the analogs to be tested. The total activity of PKC obtained with 2.5 μM OAG varied among preparations of PKC; therefore, data are expressed as percent of activity with 2.5 μM OAG and are the average of six separate determinations. Panel A, •—•, AAG(16:0); o—o, AAG(18:1). Panel B, •—•, AEG; o—o, AMG.

We chose to use 2.5 μM OAG for further experiments because this concentration resulted in an easily measured amount of stimulation, about 60% of maximum. By using a suboptimal concentration of OAG we could determine if the analogs tested were stimulatory or inhibitory.

When the alkyl analog AAG(16:0) was added in the presence of OAG it caused a concentration-dependent inhibition of PKC activity (Figure 2A). To determine if alkyl chain length or unsaturation affected the activity, we tested AAG(18:1) and found that it was also inhibitory (Figure 2A). Thus the AAG analogs are active PKC inhibitors and small changes in the alkyl chain length or the addition of a double bond in the alkyl chain do not affect activity.

The dietherglyceride analogs AMG and AEG were also tested and found to cause a concentration-dependent inhibition of OAG-stimulated PKC activity (Figure 2B). The diether analogs were not significantly different in activity indicating that small changes in the alkyl chain length at position 2 do not change the effectiveness of the compounds. In addition, the alkylacetyl analogs and the dialkyl analogs were not significantly different all demonstrating a half-maximal inhibition at approximately 20 μM. In order to further determine the effects of the inhibitors on PKC activation by OAG, the concentration dependence of activation was determined in the presence of AAG

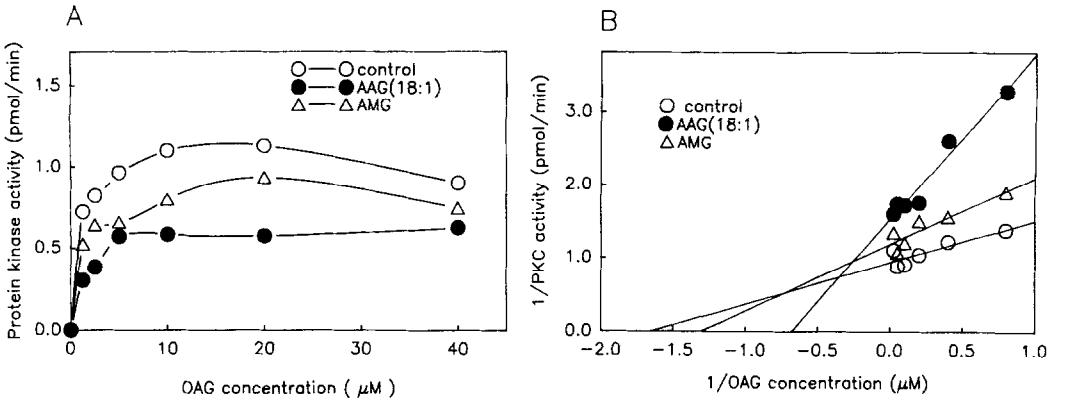

Fig. 3. Effect of alkylglycerides on PKC stimulation by diacylglycerides. PKC activity was measured in the presence of phosphatidylserine and Ca^{2+} and the indicated concentration of OAG. PKC activity is defined as total activity minus the activity in the absence of OAG. The data presented are the average of triplicate determinations from one of four similar experiments. o—o, control (no inhibitors); •—•, AAG (18:1) 5 μM; Δ—Δ, AMG, 5 μM).

(18:1) and AMG. Both inhibitors caused an increase in the apparent K_m for OAG and a decrease in the V_{max} of the enzyme. (Figure 3). These alterations are apparent when the data are plotted as the double-reciprocals (Figure 3). Therefore, the alkylglycerides are competitive with OAG for activation of the enzyme and appear to have other inhibitory effects yet to be defined.

Discussion

Alkyl-linked glycerides are produced by phospholipase C hydrolysis of PC after cell stimulation by the tumor promoter TPA (2). The function of alkyl-linked lipids is largely unknown; however, many cell types contain a significant proportion of alkyl-linked molecular species of PC. In human neutrophils 46% of the PC is the 1-O-alkyl species (19). This class of PC is also enriched in arachidonic acid and has been shown to serve as a precursor for both platelet activating factor and arachidonic acid metabolites (20). In HL-60 cells 27% of the PC is the 1-O-alkyl species (M. C. Chabot, personal communication) thus phospholipase C degradation of PC could result in the production of both 1-O-alkyl and diacyl species of diglyceride. Whether the phospholipase C that degrades PC is selective for either the alkyl or diacyl species of PC remains to be determined. However, it is clear that alkylacyl glycerides are produced. This is in contrast to the hydrolysis of

phosphatidylinositol by phospholipase C which yields diacylglycerol species.

Alkyl-linked glycerides have been shown to have biological effects different from those of diacylglycerols. Alkylacetylglycerol, AAG(16:0), in contrast to the diacylglycerol, OAG, was shown to stimulate HL-60 cell differentiation after a single application (11). Later studies have shown that although synthetic diacylglycerol analogs stimulate PKC activity in HL-60 cells, they are not effective differentiating agents (21,22). In addition, bryostatin 1 has been shown to stimulate PKC in HL-60 cells without inducing differentiation (23). These data indicate that stimulation of PKC activity is insufficient to induce differentiation.

Our studies presented here show that the alkyl-linked glycerides are inhibitors of PKC. Therefore, the observation that AAG(16:0) causes cell differentiation similar to that induced by the PKC-stimulating TPA appears paradoxical. However, since alkylacylglycerols are produced after cell stimulation by TPA, the alkylglycerides may be the functional second messenger in TPA-induced differentiation. It is of interest to note that other agents which inhibit PKC have also been shown to inhibit HL-60 cell growth and promote differentiation (13,24). Thus the alkylglycerides may be involved in a temporal sequence of PKC activation and inhibition which results in HL-60 cell differentiation. The observation that PKC activity can only be blocked by approximately 60% may indicate that different isozymes of PKC (25, 26) have differential sensitivity to activation by diacylglycerol or inhibition by AAG. Also the mixed inhibition kinetics observed with AAG and AMG may indicate more than one binding site. Overall these studies indicate a complex regulation of PKC by the different molecular species of diglycerides produced in stimulated cells. The synthetic dialkylglycerides AMG and AEG which we have identified should serve as interesting probes to determine the effects of alkyl-linked glycerides in this regulatory system.

Acknowledgments

This research was supported by a grant from the National Institutes of Health (CA-43297) and by a grant from the American Cancer Society (RD-262).

its mitogenic action, is not mediated by autocrine PDGF production. This conclusion is further strengthened by other observations: (i) addition of neutralizing anti-PDGF antibodies does not inhibit the mitogenic effect of TGFβ; (ii) TGFβ does not induce EL2 cells to form colonies in agar, while PDGF induces the growth of EL2 cells in semi-solid medium (10).

In conclusion, these studies indicate that a variety of molecular mechanisms may mediate the induction of c-fos expression by different growth factors in various types of fibroblasts. The spike of fos RNA and protein expression in EL2 or NIH-3T3 fibroblasts following EGF or PDGF treatment is mediated by transcriptional activation of fos gene and efficient fos RNA translation, followed by early blockade of c-fos transcription and rapid degradation of fos RNA and protein. In sharp contrast to this phenomenon, the sustained induction of fos RNA and protein in EL2 fibroblasts exposed to TGF β may be tentatively attributed to prolonged fos gene transcription and RNA translation and/or stabilization of fos RNA and protein (28). Conversely, the sustained fos RNA induction in NIH-3T3 fibroblasts treated with TGFβ, uncoupled with detectable fos protein(s) level, indicates prolonged activation of fos gene transcription and/or stabilization of fos RNA with concomitant blockade of efficient translation. Since the employed dosage of TGFβ induces proliferation of EL2 cells, but inhibits the growth of NIH-3T3 fibroblasts, it is suggested that the efficient translation of fos RNA in the former line is linked to and possibly mediates at least in part the proliferative response of EL2 fibroblasts to TGFβ.

It is hence apparent that a variety of molecular mechanisms, acting at transcriptional, post-transcriptional and translational level, may underlie fos gene expression induced by different growth factors in fibroblast lines. Particularly, efficient or blocked translation of fos RNA after TGFβ treatment might directly correlate with the growth stimulus or inhibition exerted by this factor in different lines.

ACKNOWLEDGEMENTS

We are grateful to T. Curran for c-fos polypeptide antiserum and to M. Sporn for the generous gift of highly purified TGF β . We thank P. Alessandri for typing the manuscript. E.L. is fellow of the "Associazione Italiana per la Ricerca sul Cancro". This work was supported in part by grants from CNR, Rome, Progetti Finalizzati Oncologia", (No. 86.02683.44 to G.B.R. and 86.00699.44 to C.P.), and "Ingegneria Genetica" (No. 86.00101.51 to G.B.R. and 86.00099.51 to C.P.).

REFERENCES

1. Gonda, T.J. and D. Metcalf. 1984. Nature (London) 310: 249-251.
2. Greenberg, M.E. and E.B. Ziff. 1984. Nature (London) 311: 433-437.
3. Kelly, K., B.H. Chocran, C.D. Stiles and P. Leder. 1983. Cell 35, 603-610.
4. Deschamps, R.L. F., Mitchell, F. Meijlink, W. Kruijer, D. Schibert and I.M Verma. 1985. Cold Spring Harbor Symposia on Quantitative Biology, Vol. L, C.S.H. Laboratory. 733-744.
5. Mitchell, R.L., L. Zokas, R.D. Schreiber and I.M. Verma. 1985. Cell 40: 209-217.