Diacylglycerol Kinase ε, but Not ζ, Selectively Removes Polyunsaturated Diacylglycerol, Inducing Altered Protein Kinase C Distribution in Vivo*

(Received for publication, February 24, 1999, and in revised form, August 24, 1999)

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Porcine aortic endothelial cells have previously been shown to contain particularly high basal levels of polyunsaturated diacylglycerol (DAG) together with a very high degree of membrane-associated protein kinase C (PKC), which is largely insensitive to further activation (Pettitt, T. R., Martin, A., Horton, T., Liossis, C., Lord, J. M., and Wakelam, M. J. O. (1997) J. Biol. Chem. 272, 17354–17359). To investigate the possibility that the high polyunsaturated DAG levels were constitutively activating PKC, we transfected porcine aortic endothelial cells with two different forms of human diacylglycerol kinase, ε and ζ. In vitro, the former is specific for polyunsaturated structures, whereas the latter shows no apparent selectivity. Overexpression of DAGKε specifically reduced the level of polyunsaturated DAG in the transfected cells while having little effect on the more saturated structures. It also caused the redistribution of PKCε and ε from the membrane to the cytosol. Overexpression of DAGKε caused a general reduction in DAG levels but had little effect on PKC distribution. These results for the first time show that DAGKε specifically phosphorylates polyunsaturated DAG in vivo and that in so doing it regulates PKC localization and activity. This provides support for the proposal that it is the polyunsaturated DAGs that function as messengers and convincing evidence for DAGKε being a physiological terminator of DAG second messenger signaling.

Agonist stimulation of cells often induces the rapid and transient phospholipase C (PLC)1-catalyzed generation of polyunsaturated sn-1,2-diacylglycerol (DAG) from phosphatidylcholine and polyunsaturated sn-1-alkenyl-2-acylglycerols. These second phospholipase C substrates, we transfected porcine aortic endothelial (PAE) cells with human DAGKε and DAGKζ, then examined the effect on DAG composition and PKC distribution. Cells transfected with DAGKε have significantly reduced polyunsaturated DAG as compared with the very high levels seen in the parental cells (10), and there is a concomitant redistribution of PKC from the membrane to the cytosol. Transfection with DAGKζ, a form with no known species selectivity (7, 11), did not alter the DAG species profile and had little effect on PKC distribution.

**EXPERIMENTAL PROCEDURES**

Materials—All solvents were of Analytical or HPLC grade from Fisher. Lipid standards were purchased from Avanti Polar Lipids Inc., Alabaster, AL. Other chemicals were from Sigma-Aldrich. Cell culture reagents were purchased from Life Technologies, Inc. The eDNA for DAGKε was cloned into pcDNA1/Neo (Invitrogen), whereas that for DAGKζ was cloned into pcDNA3.1/Zeo (+) (Invitrogen); they were generously provided by Drs. M. K. Topham and S. M. Prescott, Eccles Institute of Human Genetics, University of Utah, Salt Lake City, UT. DAGK antibodies raised in rabbit as described previously (11) were also

* The work was funded by The Wellcome Trust. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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1 The abbreviations used are: PLC, phospholipase C; DAG, sn-1,2-diacylglycerol; PA, phosphatidic acid; PIP₂, phosphatidylinositol 4,5-bisphosphate; LPA, lyso phosphatidic acid; DAGK, diacylglycerol kinase; PKC, protein kinase C; PAE, porcine aortic endothelial (cells); TPA, 12-O-tetradecanoylphorbol-13-acetate; HPLC, high performance liquid chromatography.
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supplied by Drs. Topham and Prescott. Mouse monoclonal PKCα and ε antibodies were obtained from Transduction Laboratories.

PAE cells were cultured in Ham's F-12 nutrient mixture containing Glutamax, 10% fetal calf serum, penicillin, and streptomycin and used upon reaching 70–80% confluency. Most cells were serum-starved for 24 h before use. Transfection was by electroporation. Geneticin was (100 μg/ml) used to select clones expressing human DAGKε, whereas zeocin (100 μg/ml) was used to select clones expressing human DAGKζ.

Cell Stimulation and Lipid Extraction—Cells were washed twice with phosphate-buffered saline then incubated for 15 min in Ham’s F-12 containing 20 mM HEPES, pH 7.4, and 0.1% bovine serum albumin (fraction V; Sigma) at 37 °C before stimulation with 10 μM sn-1,18:19,9-lyso-phosphatidic acid (LPA) where described. Incubations were terminated by aspiration of the media followed by the addition of ice-cold methanol and 1 μg of 1,2,12/1,2,12,0 DAG internal standard. Lipids were extracted, and diradylglycerols were derivatized and analyzed by HPLC as described previously (12, 13). Data are expressed as means ± S.D., where n = 3–6.

Inositol Phosphate Accumulation Assay—Cells were labeled with myo-[2-3H]inositol (0.5 μCi/ml) in isositol-free medium for 48 h and stimulated in the presence of 20 mM LiCl with vehicle or LPA (10 μM), and the inositol phosphates were analyzed as described previously (14).

Western Blotting of Diacylglycerol Kinase and Protein Kinase C Isozymes—PAE cells stimulated with LPA (10 μM) for 20 s and 5 min where described were washed in ice-cold phosphate-buffered saline and harvested in 20 mM HEPES, pH 7.4, containing 1 mM phenylmethyl-sulfonyl fluoride and 20 μg/ml leupeptin. The samples were frozen at −70 °C overnight, thawed, and sonicated for 20 s in a bath sonicator, and membranes and cytosol were separated by centrifugation (30,000 × g, 20 min). The membranes were washed once. The cytosolic proteins were concentrated by precipitation with 12.5% trichloroacetic acid (4 °C, 1 h) and centrifugation (13,000 × g, 10 min), and the pellet was washed with 200 μl of ice-cold acetone. The cytosolic and membrane proteins were solubilized by boiling in 75 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 4 mM urea, 5% β-mercaptoethanol, bromophenol blue for 5 min, then cell fraction equivalents were separated by electrophoresis on 7.5% SDS-polyacrylamide minigels. After transfer onto polyvinylidene difluoride membranes (Immobilon-P; Millipore), the membranes were washed in ice-cold phosphate-buffered saline then incubated for 15 min in Ham’s serum starvation for 48 h (starved from approximately 30% of the parental cells grew faster than the DAGK clones with doubling times of 21, 26, and 36 h (based on cell numbers after different culture times) for parental, DAGKε clone 7, and DAGKζ clone 7 cells, respectively. The growth of both the parental and transfected PAE cells was largely insensitive to serum starvation for 48 h (starved from approximately 30% confluency), with only a 20% increase in cell doubling time. The DAGK clones have a greatly increased propensity to align into ordered swirls when fully confluent (Fig. 2). The degree of order varied between clones but appeared to correlate with the level of DAGK expression; those expressing more enzyme showed greater alignment. The DAGK clones always showed this arrangement at confluency. The DAGK clones were more variable, sometimes showing ordered alignment into swirls and sometimes not, whereas the parental cells only occasionally showed a partial ordering. This variability with the parental and DAGK clones may relate to differences in the relative concentrations of cytokines and growth factors in different batches of cell culture serum and how these affect the DAG and/or PA signaling pathways modulated by DAGK.

PAE cells contain high levels of polyunsaturated DAG under resting conditions, and these remain largely insensitive to further stimulation (10). If DAGKε had the same specificity in vitro, overexpression of active enzyme would be expected to reduce these levels. Thus, total lipid extracts were prepared, derivatized with 3,5-dinitrobenzoyl chloride, and separated by HPLC to resolve the different DAG molecular species. Fig. 3 shows a comparison of DAG profiles from one DAGKε clone and

FIG. 1. Expression of DAGKε and DAGKζ. Total cell lysates from parental and transfected PAE cells were separated on 7.5% SDS-polyacrylamide gels and analyzed by Western blotting. The DAGK size is marked. Other clones gave similar results.

RESULTS

Expression of DAGKε and DAGKζ in parental PAE cells and DAGK-transfected clones was examined by Western blotting. Although the parental cells express both enzymes, the clones showed enhanced levels with 2–4-fold increases in DAGKε (64 kDa) and 5–10-fold increases in DAGKζ (115 kDa; Fig. 1). Preparation of cytosolic and membrane fractions revealed that these enzymes were restricted to the membrane fraction both in resting and LPA-stimulated cells (data not shown). The identifications of the immunoreactive bands at approximately 90 and 100 kDa in the DAGKζ blot remain unclear but may be cleavage products of the parent 115-kDa protein.

To the immunoprecipitate was added 5 μl of PKC substrate (either histone type IIIs or neurogranin fragment, amino acids 28–43; 1 mg/ml; Sigma) in kinase buffer, 10 μl of lipid micelle mix (500 μM phosphatidylserine, 100 μM mixed DAG, 0.5% Triton X-100 in kinase buffer), and 5 μl of kinase buffer (or 5 μM tetracosanoyl-phorbol acetate in kinase buffer for the positive controls). 10 μl of 100 μM ATP containing 0.5 μCi of [α-32P]ATP was added to start the reaction. After incubation (1 h, 30 °C), the reaction was stopped with 20 μl of 5% acetic acid. The phosphorylated substrate was separated by electrophoresis on 17.5% SDS-polyacrylamide gels for histone, whereas with the neurogranin fragment each sample was spotted onto PS1 cation exchange paper (Whatman), air dried, washed 4 × 10 min with 500 ml of 0.5% phosphoric acid and washed once with 95% ethanol. The 32P-phosphorylated substrate was detected and quantified using a Molecular Dynamics PhosphorImager.

PAE cells contain high levels of polyunsaturated DAG under resting conditions, and these remain largely insensitive to further stimulation (10). If DAGKε had the same specificity in vitro as in vivo, overexpression of active enzyme would be expected to reduce these levels. Thus, total lipid extracts were prepared, derivatized with 3,5-dinitrobenzoyl chloride, and separated by HPLC to resolve the different DAG molecular species. Fig. 3 shows a comparison of DAG profiles from one DAGKε clone and
DAGK overexpression. Table I. Overexpression of DAGK but little change in more saturated species, as summarized similar results with greatly reduced polyunsaturated DAG lev-

saturated structures, particularly 18:0/20:3n-9 and 18:0/20:

dinitrobenzoyl-derivatized, and separated into molecular species by reverse-phase HPLC. Other DAGK clones give a similar DAG profile. See Table I for peak identities. *, peaks greatly reduced by overexpres-

sion (data not shown). Total PKC activity was generally similar in the parental and

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DAGK clones, both, under basal and phorbol ester-stimulated conditions (Fig. 6A). For PKCe, basal activity was highest in the DAGKz clones and lowest in the parental cells (Fig. 6B). Although not statistically significant, the data also suggested a small increase in stimulatable PKCe activity, particularly in the DAGKz clones. Since 500 nM TPA is believed to maximally activate all DAG-sensitive PKCs in vitro, the activity seen in the presence of phorbol ester is taken to correlate with total PKC content in the immunoprecipitates. Thus, in Fig. 6, C and D, the basal PKC activity is expressed as a percentage of maximal activity (+TPA). This gives an approximation to the relative PKC specific activities under resting conditions. DAGK overexpression had essentially no effect on basal PKCa activity, which remained at approximately 50% maximal. In contrast, PKCe activity was substantially increased, particularly in the DAGKz clone, where the protein kinase was almost fully activated under basal conditions.

**DISCUSSION**

DAGK is a ubiquitous enzyme with roles in phospholipid synthesis and cell signaling. Most studies to date have reported the purification, structural identification and, in vitro characterization of novel isoforms. The data presented here provide the first demonstration that DAGK can modulate DAG levels in cells. In addition, the results show the selective modulation of DAG species levels by a particular isoform, DAGKz, but not by

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**TABLE I**

| Peak | 1,2-Diacylglycerol species | Parental | | DAGKz Clones |
|------|-----------------------------|----------|-----------------------------|-----------------------------|
|      | pmol/10^7 cells | % | pmol/10^7 cells | % |
| 1 | 18:1n-9/20:5n-3 | 31 | 0.6 | 6 | 0.2 |
| 2* | 16:0/20:5n-3 | 61 | 1.1 | 3 | 0.1 |
| 3 | 16:0/22:6n-3, 14:0/18:2n-6 | 67 | 1.2 | 53 | 1.6 |
| 4 | 14:0/16:1, 16:1/16:1 | 75 | 1.4 | 41 | 1.2 |
| 5 | 14:0/14:0, 18:1n-9/20:4n-6, 14:0/20:3n-9 | 55 | 1.0 | 39 | 1.2 |
| 6* | 16:0/20:4n-6 | 150 | 2.8 | 16 | 0.5 |
| 7 | 20:3/20:3 | 56 | 1.0 | 19 | 0.6 |
| 8* | 18:1n-9/18:2n-6, 16:1/18:1n-9, 18:0/20:5n-3 | 677 | 12.6 | 104 | 3.1 |
| 9 | 16:0/16:1, 14:0/18:1n-9, 16:0/18:2n-6 | 314 | 5.8 | 444 | 13.3 |
| 10 | 14:0/16:0, 16:0/20:3n-6 | 282 | 5.2 | 318 | 9.5 |
| 11 | 16:0/20:3n-9, 18:0/22:5n-3 | 116 | 2.2 | 38 | 1.1 |
| 12* | 18:0/20:4n-6 | 855 | 15.9 | 59 | 1.8 |
| 13 | 18:1n-9/18:2n-6, 16:1/18:1n-9, 18:0/20:5n-3 | 480 | 8.9 | 290 | 8.6 |
| 14 | 16:0/18:1n-9, 16:0/20:3n-6, 18:0/16:1 | 467 | 8.7 | 843 | 25.2 |
| 15 | 16:0/18:0, 14:0/18:0 | 773 | 3.2 | 406 | 12.1 |
| 16* | 18:0/20:3n-9 | 748 | 13.9 | 46 | 1.4 |
| 17 | 18:1n-9/20:1n-9 | 19 | 0.4 | 34 | 1.0 |
| 18 | 16:0/20:1n-9 | 13 | 0.2 | 48 | 1.4 |
| 19 | 18:0/18:1n-9 | 278 | 5.2 | 155 | 4.6 |
| 20 | 16:0/18:0 | 91 | 1.7 | 133 | 4.0 |
| 21 | 18:0/18:0 | 64 | 1.2 | 27 | 0.8 |
| Total | nmol/10^7 cells | | 5.4 | 3.4 |

* Molecular species marked in Fig. 3, which are significantly reduced in DAGKz-transfected PAE cells (p < 0.01).
nM TPA is believed to maximally activate all DAG-sensitive PKCs and PKCε localizations might contribute to the differential effect of the two DAGK forms on PKC distribution. If DAGKε and the PKCs are localized to different compartments, then DAGK modulation of local DAG levels may not affect the PKC found elsewhere in the cell. In contrast, co-localization would be expected to have a larger effect on PKC activation.

The activity data for PKCα (Fig. 6, A and C) support the idea that although different amounts of PKC may be at the membrane under basal conditions, to maintain homeostasis the cell only needs a certain level of kinase activity and, thus, modulates it accordingly, resulting in similar activities for the parental and DAGK-transfected cells. The activity in the presence of phorbol ester indicates that the three cell lines have similar total levels of PKCs. In contrast, the data for PKCε suggest that there is more enzyme in the DAGK clones (Fig. 6D). This agrees with the Western blot data where more PKCε was detected in the transfected cells (Figs. 4 and 5). The increased kinase activity in the immunoprecipitates under basal conditions may partly reflect these elevated enzyme levels, although the PKCε-specific activity appeared to be higher than in the parental cells, particularly for the DAGKε clones (Fig. 6D).

However, since the enzyme had to be immunoprecipitated and then assayed in vitro, introducing a variety of nonphysiological changes, we would argue that the PKC redistribution data are a more reliable measure of PKC activation in vivo, although not necessarily of kinase activity. In keeping with this, the increase in PKCε protein observed in the DAGK transfectants presumably reflects a reduction in the membrane polyunsaturated DAG concentration reducing the down-regulation of enzyme levels.

The molecular size of DAGKε calculated from its nucleotide sequence is 103.9 kDa, but as reported previously, it runs slightly slower than this on SDS-polyacrylamide gels (11). COS-7 cells transfected with the DAGKε gene expressed a doublet of 114 and 117 kDa as did nontransfected human glioblastoma-derived A-172 cells (11); however, both parental and transfected PAE cells expressed a band of approximately 115 kDa together with variable amounts of possible cleavage products at approximately 90 and 100 kDa (Fig. 1). Tissue-specific splice variants may be common since a 130-kDa form has been found in human skeletal tissue, although its properties appear identical to those of the 114-kDa form (15).

The higher DAGK protein expression achieved with the DAGK clones (5–10-fold above parental) as compared with the DAGKε clones (2–4 fold) suggests that the latter isozyme is probably more toxic to the cell, causing cell death when present at slightly elevated levels. This is a common problem with overexpressing lipases or other lipid-metabolizing enzymes, since only small changes can cause major membrane damage and resultant cell death. This sensitivity is likely to be greatest for enzymes involved in lipid signaling. To maintain membrane integrity and capacity for signaling, PIP$_2$, the substrate for PLC-catalyzed polyunsaturated DAG messenger generation, must be rapidly resynthesized. The first step, catalyzed by DAGK, terminates the polyunsaturated DAG signal by forming polyunsaturated PA. This is the preferred substrate for at least one form of CDP-DAG synthase (16), generating CDP-DAG (or CMP-PA), which is then utilized by phosphatidylinositol synthase for phosphoinositide synthesis (17). Sequential phosphorylation then regenerates PIP$_2$. Both DAGKε (18) and rat brain CDP-DAG synthase (16) are strongly inhibited by PIP$_2$ in vitro, providing a potential negative-feedback mechanism to regulate phosphoinositide generation. Overexpression of DAGKε would alter this balance since enhanced removal of polyunsaturated DAG would reduce the PKC activation, which negatively regulates PLC activity (13), potentially leading to sustained PIP$_2$ hydrolysis. This would deplete PIP$_2$ levels, releasing PIP$_3$ inhibition of CDP-DAG synthase and DAGKε as the cell attempts to replenish phosphoinositide. As a result the cell may become locked into an energy-draining futile cycle, which if uncontrolled, would ultimately kill it.

DAGK overexpression, particularly DAGKε, induced a no-
noticeable change in cell morphology at confluency (Fig. 2) possibly through effects on membrane fluidity caused by the reduction in total diradylglycerol levels. However, the observation that DAGKz is found at high levels within the nucleus and has a myristoylated alanine-rich C kinase substrate homology region that can act as a nuclear localization sequence (15, 19, 20) suggests that its effects maybe exerted primarily within this organelle. Phosphorylation of this domain by PKCa or PKCγ has been shown to cause DAGKz translocation out of the nucleus and away from its presumed substrate, nuclear DAG (20). Blocking this translocation by PKC down-regulation or overexpression of DAGKz in the nucleus increased the cell cycle time (20). A nuclear localization would fit with our observations that DAGKz is associated almost entirely with the membrane fraction from PAE cell lysates, since this would contain ruptured nuclei; also, that the cell doubling time was longest for the DAGKz clones. Recent work with mouse DAGKz, which shows a 95% homology with the human form, found that expression is regulated both temporally and spatially during embryonic development and correlates with the development of sensory neurons and regions undergoing apoptosis (21), suggesting a role for this enzyme in gene regulation. Thus its overexpression may lead to an abnormal expression of other genes and hence morphological change, possibly of the type observed here.

In conclusion DAGKe, but not DAGKz, modulates PKCa and ε activation by specifically regulating the endogenous levels of polyunsaturated-signaling DAG and, by generating polyunsaturated PA, initiates the resynthesis of the parent phosphoinositide.

Acknowledgments—We thank Drs S. M. Prescott and M. K. Topham for the generous donation of DAGK cDNA and antisera, which made this work possible, and for their helpful comments.

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