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Note

Measurement of intracellular diacylglycerols by acetylation and thin-layer chromatography

J.A. HOSKINS* and C.E. EVANS

MRC Toxicology Unit, Woodmansterne Road, Carshalton, Surrey SM5 4EF (U.K.)

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The hydrolysis of the cell membrane phospholipids such as phosphatidylcholine and phosphatidylinositol 4,5-diphosphate by phospholipases to produce various 1,2-diacylglycerol species is believed to be an integral part of intracellular signalling. That is the chain of chemical reactions that follows the interaction of a primary messenger with a cell receptor and is known generally as the second messenger system. This is the means by which communication is established from outside the cell with the cell nucleus. The diacylglycerols (DAGs) act within the cell by activating the membrane-bound protein kinase C, a calcium-dependent enzyme responsible for a number of intracellular reactions [1].

Underivatised DAGs have been isolated and/or measured by liquid chromatography [2] and thin-layer chromatography (TLC) [3,4] using silica gel but much of the published work describes the analysis of derivatised compounds. In the cell DAGs derive from phospholipids and for analysis they have been reconverted into this form using a fluorescent tag in situ [5] or in vitro [6]. They have been analysed following conversion to an urethane [7,8]. Esters of DAGs have been analysed by both TLC [9] and gas chromatography–mass spectrometry [10]. No single derivative, or analytical method, has emerged as the one of choice. In the small literature devoted to this class of compounds methods of isolation and analysis have been specific to the task in hand in almost every piece of published work. The study presented here is no exception: we required a method of analysis which could measure the relative levels of DAGs in about a million P388D1 or C3H10T1/2 cells and which could

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also be adapted to a qualitative analysis at a later date. DAGs derive from minor constituents in the cell membrane and to achieve the sensitivity required to analyse the small quantity of material we acetylated the DAGs with radiolabelled acetic anhydride and separated the labelled acetyldiacylglycerols from other lipids by TLC. Acetylation is a commonly used technique for the derivatisation of alcoholic hydroxyl groups and has been used before in the isolation and analysis of DAGs [11,12].

EXPERIMENTAL

Acylglycerols

Monoacid 1,2-diacyl-*rac*-glycerols, monoacylglycerols, triacylglycerols and 12-O-tetradecanoylphorbol 13-acetate (TPA) were obtained from Sigma (Poole, U.K.).

Preparation of [^{14}C]acetic anhydride[a]

To sodium [^{14}C]acetate (Du Pont, Stevenage, U.K.) (250 μCi) were added 1 ml of cold acetic anhydride and 50 μl of cold acetyl chloride, and the mixture was kept for at least 16 h at 40°C and subsequently stored at room temperature. Complete exchange of the radioactive species would give a specific activity for the acetic anhydride of 0.024 nCi/nmol. Experiments with standards showed that exchange is essentially complete.

Preparation of derivatives

(a) From standards (e.g. Dimyristin). To 0.5 mg of dimyristin in a silanised glass tube were added 50 μl of [^{14}C]acetic anhydride and 50 μl of pyridine, and the tube then sealed and heated in a block at 70°C for 30 min. After cooling, 0.5 ml of water was added and the mixture left for 10 min. The acetylated product was extracted with 0.5 ml of hexane which, after back-extraction with 0.01 *M* sodium bicarbonate solution, was evaporated under vacuum (Speed Vac concentrator, Savant, Farmingdale, NY, U.S.A.), and the residue was dissolved in 50 μl of chloroform–methanol (9:1).

(b) From cells. Cells were grown to confluence in multi-well plates of area 10 cm^2 per well. To a cell layer in a multi-well plate were added 150 μl of 10% (w/v) trichloroacetic acid plus 1 ml of phosphate-buffered saline. Lipids were extracted with 1 ml of chloroform–methanol (2:1) and the mixture was cooled at 4°C for 1 h when [^{3}H]triolein standard and an aqueous solution of EDTA

[a]It was found preferable to prepare labelled acetic anhydride rather than use the material of commerce since the quality of this was found to be so variable. Acetic anhydride from two sources was found to have a chemical purity between 0 and 50% for six batches tested. Our preparation examined similarly, by an acetylation, had a chemical purity of 92%. A probable reason for the poor quality of the commercial samples was reaction with adsorbed water on the glass vials in which the materials were sent.

(150 μl; 5 mM) were added. The organic layer was removed and the solvent evaporated. The residue was acetylated as in (*a*) and the final residue dissolved in 50 μl of chloroform–methanol (9:1).

(c) Stimulation of diacylglycerol release by TPA [13]. P388D1 (macrophage-like) cells were treated with TPA (in acetone) to a final concentration of 10^{-7} M for different periods; the reaction was stopped with TCA and the released DAGs were measured as in (*b*).

Thin-layer chromatography

The acetylated diacylglycerols were separated using pre-coated TLC plastic plates covered with silica gel 60 (Merck 5748, BDH, Poole, U.K.). For the cell experiments, 25 μl of sample were loaded onto the plates and developed in chloroform–acetone (96:4). The separated lipids were detected by spraying with 1,6-diphenylhexatriene (10 mg per 100 ml of hexane), then cutting out the acetyldiacylglycerol band (R_F=0.69) and counting in Liquiscint (National Diagnostics, Manville, NJ, U.S.A.). When the acetylation product of 1-stearoyl-2-[^{14}C]arachidonyl-*sn*-glycerol with acetic anhydride was compared on a TLC plate to [^{14}C]acetyldiacylglycerols the radiolabel had the same R_F value in both cases and there was no other significant concentration of radioactivity in either of the lanes. This shows that the reaction proceeds to completion and that no other artefactual compounds are formed.

RESULTS AND DISCUSSION

The analysis of minor components of a highly complex heterogeneous biological matrix is usually difficult. The means employed are often to deal with the heterogeneity first by, for example, precipitation of proteins or extraction of lipids, and then employ a separation into chemical class to concentrate the species of interest. The problem may be eased in the analysis of compounds from living tissues or cultured cells by radiolabelling since the analysis of radioactivity is such a sensitive technique. A labelled compound, either the one of interest or a precursor, is supplied to the tissue or cell culture and is taken up by the system labelling the compound(s) of interest. This technique, while making possible an analysis, can be expensive if incorporation of the label is poor. In our work we wished to analyse a group of cell lipids, the DAGs, which are present only in low concentration, generally within the cell membrane.

Cell lipids may be radiolabelled by growing cells in a medium containing radioactive glycerol. This method has often been used and all the cell lipids are labelled; however, it is expensive. The expense arises because to ensure a sufficient concentration of the minor lipid components for analysis a high specific activity of labelled glycerol must be used. We aimed to reduce the expense without compromising the assay by introducing a radiolabel only at a late stage in the analysis which is otherwise unchanged. Other advantages from this ap-

proach are that most of the work-up is made in the absence of radioactivity and there is no disposal problem from spent, but still highly radioactive, culture medium.

The techniques for separating lipids from a cell system by chloroform–methanol extraction [14] followed by analysis of individual compounds [15] are well established. We extracted the lipids as described in the Experimental section, adding tritiated triolein as an internal standard. Accurate quantitation of the DAGs depends upon minimal handling loss during the stages of the work-up. The use of an internal standard corrects for any loss during the steps of the procedure. With care a good result can be obtained without using an internal standard though in general it was found to give a better calibration line by correcting for experimental error. Using standards the concentration of an acetylated DAG against recovered activity from a plate shows a linear relationship over the concentration range studied. There is no doubt that an even better analysis could be made by the use of an automated TLC scanner. Losses of material do occur when cutting up the TLC plate, and inaccuracies arise from counting a heterogeneous mixture.

The DAGs are an integral part of several intracellular messenger systems. When extracellular messenger compounds, such as catecholamines or small

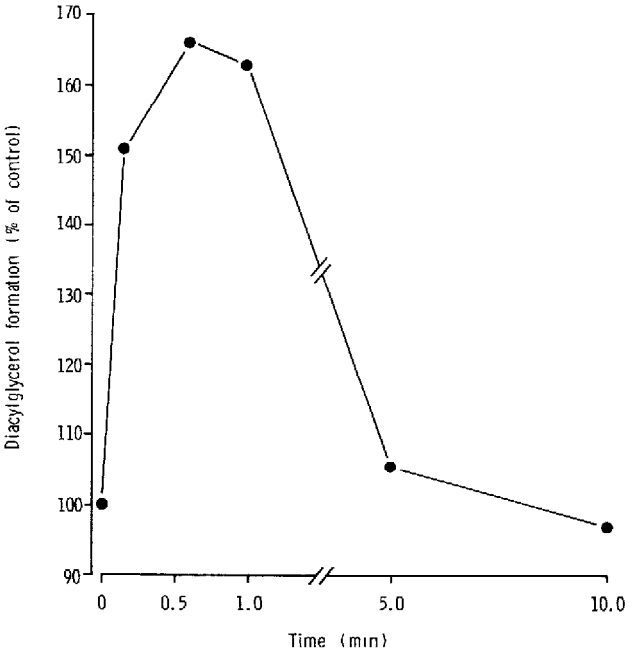

Fig. 1. Levels of DAGs generated in P388D1 cells following stimulation with 10^{-7} *M* TPA (as a percentage of control) [13]. The control level represents counts from 200 to 400 dpm depending upon the culture used.

peptides, interact specifically with cell surface receptors on a target cell their message is conveyed to the cell nucleus for action by an intracellular 'second' messenger system. DAGs may be part of such an intracellular message and result from hydrolysis of certain cell membrane phospholipids. The amount of the total cell phospholipids which can generate DAGs depend upon cell type but is around 1%. The amount of free DAGs in a cell is a measure of certain metabolic activities within the cell. In a resting cell the instantaneous levels are low. DAGs are continually being formed and removed by further metabolism, mainly phosphorylation with DAG kinase. Stimulation of the second messenger system with an external agent results in an increase in DAG levels. The time course over which this occurs and the extent to which it occurs are compound- and cell type-dependent. It has been shown [13] that certain phorbol esters, notably TPA, stimulate the transient production of DAGs. This DAG burst may last only a few minutes and be at its peak within 30 s. It can be demonstrated using the method described in this paper (Fig. 1). The result obtained agrees with published work using a culture medium containing radiolabelled glycerol.

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