Diacylglycerol generated during sphingomyelin synthesis is involved in protein kinase C activation and cell proliferation in Madin–Darby canine kidney cells

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We have investigated the effects of inhibiting sphingomyelin (SM) biosynthesis on cellular diacylglycerol (DAG) content and protein kinase C (PKC) activation during growth initiation in Madin–Darby canine kidney cells. We utilized β-chloroalanine (BCA) to inactivate serine *C*-palmitoyltransferase, the first enzyme in the sphingolipid biosynthesis pathway. This inactivation prevented growth, but did not affect viability. When the inhibitor was replaced with fresh culture medium, the cells continued their proliferation in a normal way. BCA (2 mM) inhibited [^{32}P]P_i, [^{3}H]palmitic acid and [*methyl*-^{3}H]choline incorporation into SM, but did not influence the synthesis of other major phospholipids. SM synthesis and DAG generation were decreased by 51 % and 47.6 % respectively. Particulate PKC activity was not observed in cells incubated with BCA, in contrast with a 5-fold increase in control cells. BCA inhibited 75 % of the [^{3}H]thymidine incorporation, and the cells were arrested before the S phase of the cell cycle. Moreover, exogenous D-erythrosphingosine restored SM synthesis, DAG generation and cell proliferation. These data indicate that the contribution of DAG generated during SM synthesis plays an important role in PKC activation and cell proliferation.

Key words: diacylglycerol (DAG) generation, protein kinase C (PKC) regulation, sphingomyelin synthesis.

INTRODUCTION

In the majority of cells, agonist-stimulated diacylglycerol (DAG) production is generated by the activation of several signalling pathways. Changes in DAG mass and phospholipid turnover suggest that hydrolysis of phosphatidylcholine (PC) contributes to the majority of DAG in cells [1]. Three pathways of PC metabolism are potentially linked to DAG generation: one catalysed by PC-specific phospholipase C (PC-PLC); a second pathway catalysed by PC-specific phospholipase D (PC-PLD), followed by phosphatidic acid phosphatase (PAP) activation, and the third the conversion of ceramide into sphingomyelin (SM) catalysed by PC ceramide choline phosphotransferase (SM synthase; EC 2.7.8.-). Numerous studies have investigated the generation of DAG by the PC-PLC- and PC-PLD–PAP-mediated signalling pathways [2], and the signalling role of ceramides, sphingosine and their derivatives produced through the degradation of membrane sphingolipids are currently under intense investigation [3–5]. In contrast, signalling mediated by the generation of DAG during the synthesis of SM has not been reported. We have studied the possible contribution of SM biosynthesis in the generation of DAG and subsequent protein kinase C (PKC) activation during growth in Madin–Darby canine kidney (MDCK) cells. MDCK cells were chosen because they possess DAG-sensitive conventional PKCα and β isoenzymes, which translocate from cytosol to plasma membrane upon activation with DAG [6,7].

We took advantage of the availability of three metabolic inhibitors: β-chloroalanine (BCA), D609 and fumonisin B1. BCA inhibits serine *C*-palmitoyltransferase (SPT, E.C.2.3.1.50), the first committed step of sphingolipid synthesis. SPT is a pyridoxal 5′-phosphate-dependent enzyme, and it has been shown that 5 mM BCA inhibits SPT completely in 15 min without affecting cell viability for up to 6 h [8]. β-F-Alanine inhibits sphinganine synthesis and its incorporation into sphingomyelin by at least 60 %, and fumonisin B1 is an inhibitor of ceramide synthase that decreased the SM mass by approx. 50 % [9]. In the present study, when the synthesis of SM was inhibited with 2 mM BCA, the generation of DAG was decreased and there was no PKC activation. DNA synthesis was also inhibited, suggesting that cells were arrested before S phase, probably at the G_1 to S phase transition. Fumonisin B1 (0.14–1 μM) and D609 (20–40 μg/ml) inhibited SM synthesis and decreased DAG levels to a similar extent. Our data provide evidence that DAG generated during SM synthesis participates in the activation of PKC and is required for the normal transit through the cell cycle.

MATERIALS AND METHODS

Materials

[^{32}P]P_i (carrier-free, 10 mCi/ml), [γ-^{32}P]ATP (> 5000 Ci/mmol; PB-108), [*methyl*-^{3}H]thymidine (46 Ci/mmol), [9,10(*n*)-^{3}H]palmitic acid (54 Ci/mmol), [*methyl*-^{3}H]choline chloride (60–85 Ci/mmol) and the DAG assay system were purchased from Amersham Biosciences. Fetal bovine serum (FBS), trypsin and the PKC assay kit were obtained from Gibco; Dulbecco's modified Eagle's medium (DMEM) was from Microlab; BCA, fumonisin B1, D609, *o*-phthalaldehyde and other chemicals were from Sigma; organic solvents were obtained from Merck, and scintillation liquid was from New England Nuclear.

Abbreviations used: BCA, β-chloroalanine, DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MDCK, Madin–Darby canine kidney; PAP, phosphatidic acid phosphatase; PC, phosphatidylcholine; PC-PLC, PC-specific phospholipase C; PC-PLD, PC-specific phospholipase D; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PKC, protein kinase C; SM, sphingomyelin; SPT, serine *C*-palmitoyltransferase.

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Cell cultures

MDCK cells between passages 65–75 were obtained from the American Type Culture Collection and were a gift of Dr J. L. Reyes (Department of Physiology, Centro Investigaciones y de Estudios Avanzados, México). Cells were cultured in complete medium [DMEM supplemented with 10 % (v/v) FBS, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine] in a humidified air incubator containing 5 % CO_2 at 37 °C, and cells were grown to confluence (5–6 days). The culture medium was removed and cells were trypsinized for the minimum time required for the cells to detach from the culture dish. Cells were then washed and resuspended in PBS and viability was assessed by the Trypan Blue exclusion method. All experiments were initiated with cell suspensions obtained from confluent cultures. After trypsinization, cells were washed once with PBS/DMEM (1:1, v/v), collected by gentle centrifugation, resuspended in fresh medium and allowed to adhere to 100 mm tissue-culture dishes (Corning) at (5.5–6.0) $\times 10^6$ cells/8 ml of complete medium and incubated at 37 °C in 5 % CO_2.

Cell growth

MDCK cells (5×10^5 cells) were seeded in complete medium on 35 mm plastic plates with or without BCA at various concentrations. At the indicated times, cells were trypsinized and the cell number was counted with a haemocytometer and cell viability was checked. Cell numbers were derived from the mean of a minimum of two independent experiments.

Labelling and analysis of phospholipids

Pulse and steady-state labelling of phospholipids were performed. Lipids were extracted using the methanol stop procedure [10]. The addition of methanol prior to scraping the cells from the dish was shown to stop cellular metabolism and permit accurate quantification, and was compatible with cultures grown on plastic. Directly after removal of the culture dishes from the incubator, the complete medium was aspirated off as completely as possible, the cells were washed twice with PBS and 1 ml of methanol was added. Cells were scraped off the dish and transferred to a 13 mm $\times$ 100 mm glass tube. Dishes were washed once with 1 ml of methanol, which was also transferred to the tubes. Phases were split by the addition of chloroform (1 ml) and water to obtain a chloroform/methanol/water ratio of 5:10:4 (by vol.). The monolayer was vortex-mixed and left at room temperature. After 1 h, the cellular debris was pelleted by centrifugation and the supernatant transferred to another tube (16 mm $\times$ 125 mm). The pellet was re-extracted with 3.8 ml of chloroform/methanol/water (5:10:4, by vol.) for 1 h. After centrifugation, the supernatants were combined. Phospholipid phosphorus (nmol of P_i present in the chloroform phase) was determined by the method of Ames [11], and labelled lipids in the organic phase were resolved by two-dimensional TLC on silica gel G plates and developed in chloroform/methanol/acetic acid/water (85:20:8:4, by vol.) in the first dimension and chloroform/methanol/28 % (v/v) ammonium hydroxide (81:31:5, by vol.) in the second dimension. For the separation of DAG, petroleum ether/diethyl ether/acetic acid (90:10:1, by vol.) was used. After separation, lipids were visualized by exposure of the plates to iodine vapour or after autoradiography of plates where the corresponding standards were included. The individual areas were removed from the plate and quantified by scintillation counting. Radioactivity was corrected for the amount of phospholipids.

Mass measurement of cellular phospholipids

To label phospholipids pools uniformly for steady-state labelling, cells were seeded at 6×10^6 cells (in 100 mm dishes) in complete medium containing [^{32}P]P_i (10 μCi/ml) or [^{3}H]palmitic acid (5 μCi/ml) and grown to confluence. The use of large dishes ensured that the cells had adequate room to divide several times before contact inhibition occurred. Knowing the amount (nmol) of P_i present in the organic phase and the percentage phospholipid composition in the steady-state labelled cells, the amount of each phospholipid was determined and expressed as nmol/mg of protein. The protein concentration was measured by the method of Bradford [12] using BSA as the standard. To monitor if there was any phospholipid accumulation during growth re-initiation (during the first 10 h of incubation), uniformly labelled cells were also subcultured for different times in culture media identical with that used for steady-state labelling and the levels of phospholipids and DAG were determined.

DAG mass measurements

A radioenzymic assay employing *Escherichia coli* DAG kinase (Amersham Biosciences) was used to quantify DAG production under defined mixed micelle conditions [7.5 % (w/v) *n*-octyl-β-glucopyranoside and 5 mM cardiolipin in 1 mM diethylenethyamine pentacetic acid] to solubilize the DAG. In the presence of [γ-^{32}P]ATP (> 5000 Ci/mmol), the enzyme converts DAG into [^{32}P]phosphatidic acid. The assay was performed according to the manufacturer's instructions. [^{32}P]Phosphatidic acid was extracted and subsequently separated from other lipids and residual [γ-^{32}P]ATP by TLC using phosphatidic acid (Sigma) as reference. The radioactivity of [^{32}P]phosphatidic acid was estimated by liquid-scintillation counting. The DAG/phospholipid ratios were calculated and expressed as pmol of DAG/nmol of phosphate. The use of DAG/phospholipid ratios corrects for differences in cell number and recoveries (see [10]). Ceramide levels were evaluated using the DAG kinase method.

Measurement of phospholipid turnover

Confluent cultures of MDCK cells were subcultured in DMEM alone containing 10 μCi/ml [^{32}P]P_i in the absence or presence of 2 mM BCA. At $t = 0$, the average cell number per 100 mm dish was (5.8–6.0) $\times 10^6$ cells. After 2 h, the cells were washed with PBS, and fresh medium in the absence or presence of 2 mM BCA was added. At the indicated times up until 10 h, duplicate dishes of cells were harvested by washing twice with ice-cold PBS and the lipids were extracted, separated by TLC and quantified as described above. At the 2 h pulse time, there were 226 700 c.p.m./dish in the control and 200 000 c.p.m./dish in the cells treated with BCA.

MDCK cells were also labelled with [^{3}H]choline (5 μCi/ml) in the absence or presence of BCA. At the indicated times, duplicate dishes of cells were harvested, extracted and analysed as described above.

PKC assay

MDCK cells were grown to confluence and 6.0×10^6 cells were subcultured on 100 mm tissue-culture plates containing complete medium in the absence (control) or presence of 2 mM BCA. After incubation for the indicated times, cells were washed twice with PBS, scraped from dishes in extraction buffer [20 mM Tris/HCl (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA and 25 μg/ml

each of aprotinin and leupeptin], disrupted by freeze-thawing and the cytosolic and membrane fractions were separated by centrifugation (100 000 ***g***, 1 h, 4 °C) in a Beckman L-37 ultracentrifuge. The resulting supernatant was retained (cytosolic fraction) and the pellet was resuspended in extraction buffer containing 0.5 % Triton X-100. Cytosolic and membrane fractions were assayed for PKC activity using a PKC assay kit (Gibco). The PKC assay kit is based on measurement of the phosphorylation of acetyl-myelin basic protein(4–14) as described by Yasuda et al. [13], and results are expressed as pmol of phosphate/min per 6×10^6 cells. Protein levels was corrected on the basis of protein content of the dish analysed.

Measurement of levels of sphingoid bases

Sphingosine and sphinganine (natural inhibitors of PKC) were quantified using HPLC as described by Merrill et al. [14]. Sphingosine and sphinganine standards were used for identification and quantification. Initial studies indicated that a minimum of 5×10^6 cells were required for sufficient cellular sphingosine to be accurately determined by this method.

Measurement of [^{3}H]thymidine incorporation

MDCK cells (1×10^5cells/well) were incubated in complete medium. [^{3}H]Thymidine (1 μCi/ml) was added to each well 2 h before the end of the incubation period. Cells were washed twice with ice-cold PBS and fixed with 95 % (v/v) methanol (15 min). After being washed with PBS, the cells were lysed with 0.2 M NaOH, neutralized with 0.2 M HCl and subjected to liquid-scintillation counting.

Statistical analysis

Difference between means was compared using Student's *t* test or ANOVA using the SAS computer program.

RESULTS

Effect of BCA on MDCK cell viability and proliferation

It is known that there is a concentration- and time-dependence of inhibition of long-chain base synthesis by BCA [8]. In order to explore the relevance of sphingolipid synthesis on cell proliferation, we examined the effect of BCA. Figure 1 shows that there was a concentration-dependent inhibition of cell proliferation and that 2 mM BCA resulted in a complete inhibition. After 10 h of exposure to BCA, there was no loss of cell viability (> 90 % Trypan Blue exclusion) and the cells remained adhered to the dish without alterations in cell morphology. When the inhibitor was removed and replaced with fresh medium, cell proliferation was re-activated and the cultures reached confluence after 5–6 days (results not shown).

Effect of BCA on phospholipid synthesis

Exogenous added [9,10(*n*)-^{3}H]palmitic acid was used to estimate the synthesis of phospholipids. After 2 h in complete medium in the absence (control) or presence of 2 mM BCA, MDCK cells were washed twice with DMEM to remove BCA and then were labelled with [^{3}H]palmitic acid (5 μCi/ml of culture medium) for 3.5 or 5.5 h. Labelling was started after 2 h of incubation because, at this time, the levels of endogenous sphingoid bases were drastically decreased ([9], and see below). Table 1 shows that BCA decreased the amount of [^{3}H]palmitic acid in SM in the cells pulsed for 3.5 h and 5.5 h by 45 and 49 % respectively (Table 1, columns 2 and 5). In other experiments, cells were exposed to BCA for 2 h before and for 3.5 h during the pulse. The amount of [^{3}H]palmitic acid incorporated into SM was decreased by 76 % (Table 1, column 3). Under all conditions tested, the incorporation of radiolabel into total lipid extract, PC, phosphatidylserine (PS)/phosphtidylinositol (PI) and phosphatidylethanolamine (PE) was not altered by exposure to BCA. These data indicate that BCA does not interfere with the biosynthesis of other phospholipids, at least during the first 7.5 h of incubation. Similar results were obtained with 5 μCi/ml [*methyl*-^{3}H]choline. After 7.5 h of incubation in the presence of 2 mM BCA there was a 42 % decrease in labelled sphingomyelin (380 ± 5 compared with 220 ± 17 d.p.m./nmol of lipid phosphorus) without a significant difference in labelled PC (1291 ± 10 compared with 1179 ± 130 d.p.m./nmol of lipid phosphorus). [^{3}H]Palmitic acid incorporation into DAG was decreased by 60 %.

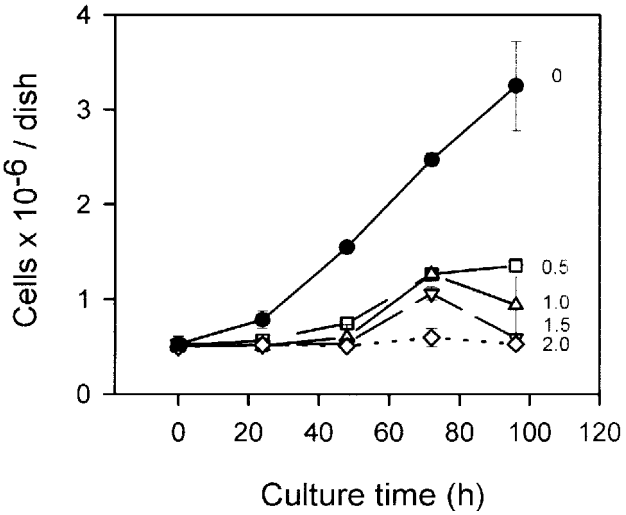

Figure 1 Effect of BCA on MDCK cells proliferation and viability

MDCK cells were seeded at a density of 5×10^4 cells/cm^2 (35 mm dish) in complete medium in the absence (control, ●) or presence of 0.5 (□), 1.0 (△), 1.5 (▽) and 2.0 (◇) mM BCA. Cell density was determined by trypsinizing duplicate dishes at the indicated times and viable cells were counted. Results are means ± S.D. of three independent experiments.

Table 1 Effects of BCA on *de novo* phospholipid synthesis

MDCK cells were incubated with 5 μCi/ml [^{3}H]palmitic acid in culture medium for 3.5 and 5.5 h, after which cellular lipids were extracted and analysed as described in the Materials and methods section. The cells were preincubated with 2 mM BCA for 2 h and washed out before the pulse (− 2 h), or BCA was present before and during the pulse (5.5 h). Values are expressed as d.p.m./nmol of phospholipid and are means ± S.D. of triplicate samples from a representative experiment. *$P < 0.05$ and **$P < 0.01$ compared with control

	Incubation time				
	5.5 h (3.5 h pulse)			7.5 h (5.5 h pulse)	
Lipid	Control	BCA (− 2 h)	BCA (5.5 h)	Control	BCA (− 2 h)
Total	21 972 ± 1502	21 460 ± 2408	21 064 ± 1557	28 727 ± 2153	25 007 ± 3488
PC	12 497 ± 1465	13 190 ± 2497	14 919 ± 1418	16 464 ± 2592	15 983 ± 2351
SM	5006 ± 658	2557 ± 66*	1213 ± 75**	5451 ± 612	3004 ± 867*
PS/PI	2746 ± 510	3389 ± 346	2864 ± 233	4274 ± 570	3578 ± 123
PE	1641 ± 97	2124 ± 328	2057 ± 53	2454 ± 367	2404 ± 290
DAG	1183 ± 322	474 ± 17*	–	–	–

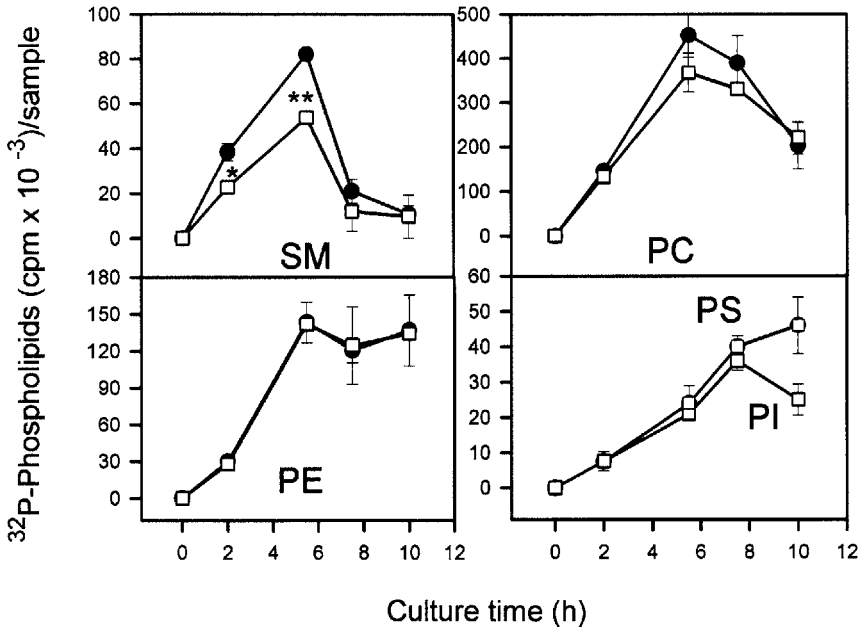

Figure 2 Effect of 2 mM BCA on [^{32}P]-labelled phospholipid turnover

MDCK cells (5.6×10^6) were seeded in complete medium (0 h time point) and pulse-labelled for 2 h with 10 μCi/ml [^{32}P]P_i in the absence (control, ●) or presence (□) of 2 mM BCA, washed twice with PBS, followed by culture medium (without isotope) with or without 2 mM BCA. At the indicated times, cells were harvested, lipids were extracted, separated by TLC and [^{32}P] incorporated into phospholipids was quantified. Results are expressed as c.p.m. $\times 10^{-3}$/sample and are means $\pm$ S.D. of duplicate dishes from five separate experiments. *$P < 0.05$ and **$P < 0.01$ compared with control.

Effect of 2 mM BCA on phospholipid turnover

We examined the effect of 2 mM BCA on phospholipid formation (2 h pulse) and lipid turnover (2–10 h chase). The incorporation of [^{32}P]P_i into phospholipids was similar in both the control and the BCA-treated cells (720 ± 77 and 615 ± 54 c.p.m. $\times 10^{-3}$ respectively, after 5.5 h). The only significant difference observed was a 35 % inhibition in the labelling of SM after 5.5 h of culture (Figure 2). The turnover of PC and PI occurred, since labelled PC decreased by 50 % (from 5–10 h) and labelled PI decreased by 31 % (from 8–10 h). Removal of the choline or phosphocholine moiety from PC, with a calculated half-life of 2–4 h when choline was used as the radiolabel, has been found to be specific for the G_1 phase of both the first and the second cell cycles [15]. Interestingly, labelled SM decreased by 75 % in 2.5 h (between 5.5 and 7.5 h of culture). To our knowledge, this early and fast SM turnover has not been observed before.

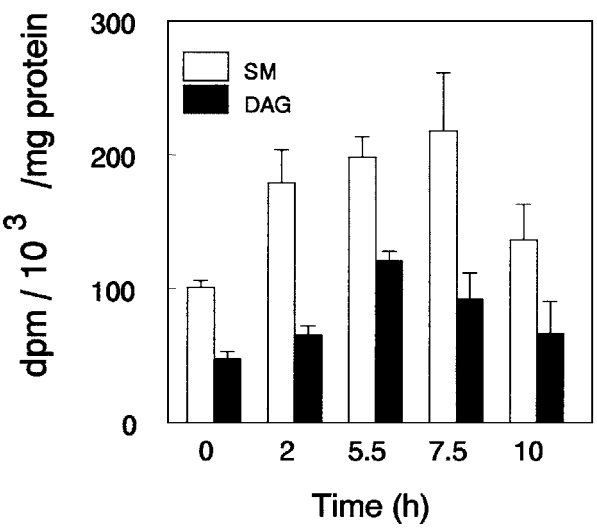

Figure 3 Time course of SM synthesis and DAG generation

Cells were prelabelled with [^{3}H]palmitic acid for 5 days, washed and $(5.5–6) \times 10^6$ cells were seeded in complete medium without label. At the indicated times, lipids were extracted and separated by TLC and label incorporated into SM and DAG was determined. The results are expressed as d.p.m. $\times 10^{-3}$ per mg of protein. Results are the means $\pm$ S.D. of triplicate determinations from a representative experiment of three separate experiments.

SM synthesis and DAG generation

To establish possible changes in the formation of DAG during the synthesis of SM and to confirm their turnover, MDCK cells were prelabelled with [^{3}H]palmitic acid to steady state, washed twice with DMEM and $(5.5–6.0) \times 10^6$ cells were seeded in complete medium without label. At the indicated times, lipids were extracted and separated by TLC, and the incorporation of [^{3}H]palmitic acid into SM and DAG was determined. As shown in Figure 3, there was a temporal correlation between SM synthesis and the generation of labelled DAG. During the first 5.5 h of incubation, SM and DAG increased approx. 2- and 2.5-fold respectively. Between 5.5 h and 10 h of incubation both SM and DAG decreased towards the levels found in confluent cells (0 h time point). Such a decrease in labelled SM could result from the activation of a reverse in SM synthesis, which has been found in MDCK cells [16], or SM degradation. This reverse in SM synthesis could explain the decrease in DAG and/or the ceramide produced. Alternatively, SM degradation generates ceramide. The ceramide produced could be utilized for the synthesis of glycosphingolipids or SM, or participate in the activation of protein phosphatases (for reviews see [5,17]).

Table 2 Influence of time of incubation on DAG levels in MDCK cells analysed by mass measurements

MDCK cells derived from confluent cultures were seeded in complete medium in plastic dishes at a cell density of (5.8–6) $\times 10^6$ cells in the absence or presence of 2 mM BCA and directly analysed for DAG content (DAG kinase) and total phospholipid phosphorus at the indicated times of incubation. Values are means ± S.D. from measurements on six separate dishes. The DAG at start was measured in confluent cells before trypsinization. –, not determined.

	DAG/phospholipid (pmol of DAG/nmol of total phospholipid)	
Time (h)	Control	BCA
Start (0 h)	18.5 ± 2.0	18.5 ± 2.0
2	59.7 ± 4.2	24 ± 2.0
5.5	68.5 ± 5.0	30 ± 2.0
7.5	51.7 ± 4.0	–
10.0	33.0 ± 3.0	16.0 ± 3.0

Effect of time of incubation and BCA on cellular DAG mass

MDCK cells [(5.8–6.0) $\times 10^6$ cells] were seeded in plastic dishes containing 8 ml of complete medium and incubated at 37 °C in the absence (control) or presence of 2 mM BCA. At the indicated times, DAG (DAG kinase) and total phospholipid phosphorus content were determined and DAG/phospholipid ratios (pmol of DAG/nmol of total phospholipid) were calculated. Table 2 shows that the amount of DAG increased 3.2 to 3.9 times after 2.0 and 5.5 h of incubation and then decreased (between 7.5 and 10 h) towards the level found in confluent cells (0 h time point). This behaviour was similar to that found in cells prelabelled with [^{3}H]palmitic acid (Figure 3). In contrast, in the BCA-treated cells, the amount of DAG measured after 2.0 and 5.5 h of incubation was decreased 2.3 times when compared with the control values.

Effect of BCA on MDCK PKC

DAG-dependent activation of PKC is associated with a change in its subcellular distribution from the cytosol to the membrane fraction. Therefore in order to know if the DAG generated during SM synthesis was involved in signalling, the activation of PKC (particulate fraction) was determined at 2 and 5 h of incubation. These time points were selected on the basis of the observed increase in DAG and SM synthesis. The PKC activity in the particulate fraction increased 5-fold in the control, whereas in the BCA-treated cells no increase was detected (Table 3). This supports further our proposal that DAG generated during SM synthesis participates in signalling (PKC activation). Addition of 100 nM PMA 30 min before harvesting increased particulate PKC activity in the BCA-treated cells.

Content of sphingoid bases in MDCK cells

Sphingoid bases are reported to be inhibitors of PKC [4] and, therefore, we determined the levels of sphingosine and sphinganine. After 2 h of incubation in DMEM, the levels of sphingoid bases were drastically decreased and, after 5 h, only 1 % of sphingoid bases determined at 0 h remained (Table 4). In cells exposed to BCA, a similar effect was observed and, since there was an inhibition of the synthesis of sphingoid bases, the decrease was even greater. These results suggest a rapid metabolic transformation following cell-cycle activation and minimize the possibility that sphingoid bases can affect PKC activation (between 2 to 5 h) in the BCA-treated cells. In fact, it has been reported that sphingosine 1-phosphate is synthesized rapidly in response to mitogenic signals, such as serum or platelet-derived growth factor ('PDGF'), in many mammalian cells [18,19]. Therefore the possibility exists that this process was also taking place in MDCK cells.

Table 3 Inhibition of PKC activation by 2 mM BCA

Results are means ± S.D. of three independent experiments performed in duplicate. PMA (100 nM) was added 30 min before harvest.

	Particulate PKC (pmol of phosphate/min per mg of protein)			
Time (h)	Control	BCA	Control + PMA	BCA + PMA
0	4.7 ± 0.5			
2	11.5 ± 1.0	4.2 ± 1.0		
5.5	24.5 ± 4.0	4.7 ± 2.0	24.3 ± 4.3	21.4 ± 0.9
7.5	3.7 ± 1.7	1.0 ± 0.5		
10.0	1.8 ± 1.5	1.4 ± 0.7		

Table 4 Effect of time of incubation and BCA on cellular concentrations of sphingoid bases

Cells were incubated in DMEM alone in the absence or presence of 2 mM BCA. Sphingoid bases were extracted and their *o*-phthalaldehyde derivates were prepared and analysed by HPLC, as described in the text. Values are means ± S.D. of five separate experiments. ND, not detected.

	Sphingosine (pmol $\times 10^6$ cells)		Sphinganine (pmol $\times 10^6$ cells)	
Time (h)	Control	BCA	Control	BCA
0	110.7 ± 23		21.0 ± 6.4	
2	4.0 ± 2.5	0.3 ± 0.3	1.06 ± 0.8	0.4 ± 0.4
5	1.0 ± 0.03	0.48 ± 0.42	0.25 ± 0.03	0.10 ± 0.01
7.5	11.7 ± 0.2	ND	6.5 ± 1.2	ND
10.0	6.0 ± 1.3	ND	3.1 ± 1.2	ND

Effect of D-erythrosphingosine on BCA-treated cells

Since BCA, which has also been used to inhibit SPT in intact cells [8], appears to inhibit various pyridoxal phosphate-dependent enzymes in addition to SPT, we examined if D-erythrosphingosine (1, 2 and 5 μM) added to the incubation medium restored the generation of DAG. Table 5 shows that 1 and 2 μM sphingosine restored the generation of DAG in the BCA-treated cells determined at 5.5 h of incubation, and 5 μM sphingosine did not increase further the amount of DAG. When 2 mM BCA was present throughout the treatment period, the addition of 1 and 5 μM D-erythrosphingosine to the culture medium resulted in a 56 and 50 % restoration of growth respectively, after 48 h of incubation. Higher concentrations of sphingosine were less efficient (34.5 % growth recoveries were observed with 10 μM, and 20 μM was cytotoxic). When added exogenously, sphingosine is a potent inhibitor of PKC, and sphingosine was also found to inhibit the activity of other enzymes, including Na^+/K^+ ATPase, calcium/calmodulin-dependent protein kinase, insulin-receptor kinase and CTP: phosphocholine cytidylyltransferase [20].

Effect of fumonisin B1 and D609 on the synthesis of SM, the generation of DAG, ceramide levels and PKC activation

The effects of fumonisin B1 (inhibitor of ceramide synthesis) [9] and D609 (inhibitor of SM synthesis) [21] on ceramide, DAG mass

Table 5 Exogenous D-erythrosphingosine restored the generation of DAG in BCA-treated cells

MDCK cells were seeded in complete medium in the absence or presence of 2 mM BCA. Duplicate dishes received D-erythrosphingosine (1, 2 and 5 μM) or ethanol (control). After 5.5 h of incubation, lipids were extracted and DAG was quantitated. Results are means ± S.D. of triplicate determinations from a representative experiment. Cells were also cultured as described above and after 48 h of incubation the viable cell number was determined. The recovery of cell growth was expressed as a percentage of growth in the absence of BCA. *$P < 0.01$ compared with control.

	DAG (pmol of DAG/nmol of phospholipid phosphorus)		Growth recovery (%)	
Treatment	Control	BCA	Control	BCA
D-erythrosphingosine				
0	73.3 ± 10	38.8 ± 2.6*	100	0
1 μM	85.8 ± 19	84.3 ± 12.2	93	56
2 μM	75.0 ± 9.2	67.1 ± 10.0		
5 μM	77.7 ± 13.0	65.8 ± 13.4	89	50

Table 6 Effect of fumonisin B1, D609 and BCA on sphingomyelin synthesis, DAG, ceramide mass levels and particulate PKC activity

MDCK cells (5.6×10^6) in complete medium were incubated with 10 μCi/ml [^{32}P]P_i for 5.5 h in the absence (control) or presence of 0.14 μM fumonisin B1, 20 μg/ml D609 or 2 mM BCA. Cellular lipids were extracted and analysed as described in the Materials and methods section. Results are means ± S.D. of triplicate determinations from a representative experiment with similar results obtained from a minimum of three independent experiments. The units for the lipids are as follows: c.p.m./samples for PC and SM; pmol/nmol of phospholipid phosphorus for DAG and ceramide; and pmol of phosphate/min per mg of protein for PKC. *$P < 0.05$ and **$P < 0.01$ compared with control.

Lipid	Control	FB1	D609	BCA
PC	102593 ± 9903	99351 ± 355	109816 ± 5372	95000 ± 7000
SM	10319 ± 868	5375 ± 584**	4745 ± 254**	5000 ± 400**
DAG	63.7 ± 7.6	34.0 ± 6*	33 ± 3.6*	30.2 ± 4.0*
Ceraminde	8.4 ± 1.9	3.6 ± 0.7**	22.5 ± 5.0*	4.3 ± 0.8**
PKC	18.1 ± 3.62	2.7 ± 0.36*	6.81 ± 1.82*	7.06 ± 0.46*

levels, PKC activity and SM synthesis were investigated. Table 6 shows that 140 nM fumonisin B1 and 20 μg/ml D609 decreased the synthesis of SM by 48–54 % and inhibited DAG generation by 47–48 %. Increasing the concentration of fumonisin B1 up to 1 μM or increasing the concentration of D609 to 40 μg/ml did not enhance further the decrease in SM synthesis and DAG generation. These results confirm the significant contribution of the synthesis of SM to the increments in DAG. Ceramide levels were decreased with BCA and fumonisin B1 and increased with D609. Particulate PKC activity was decreased in the cells treated with these compounds. In the fumonisin B1-induced inhibition of PKC observed, the accumulation of sphingoid bases must be considered [20] in addition to a decreased DAG level. The data showed that SM synthesis, DAG levels and PKC activity are closely related.

Changes in total phospholipids during growth re-initiation (first 10 h)

Since the contribution of the hydrolysis of PC and SM or their interconversion into the steady-state lipid composition may be significant, the steady state of phospholipid composition was studied after uniform labelling with [^{32}P]P_i. The basal level of phospholipids was 101.4 ± 8 nmol/mg of protein and increased only to

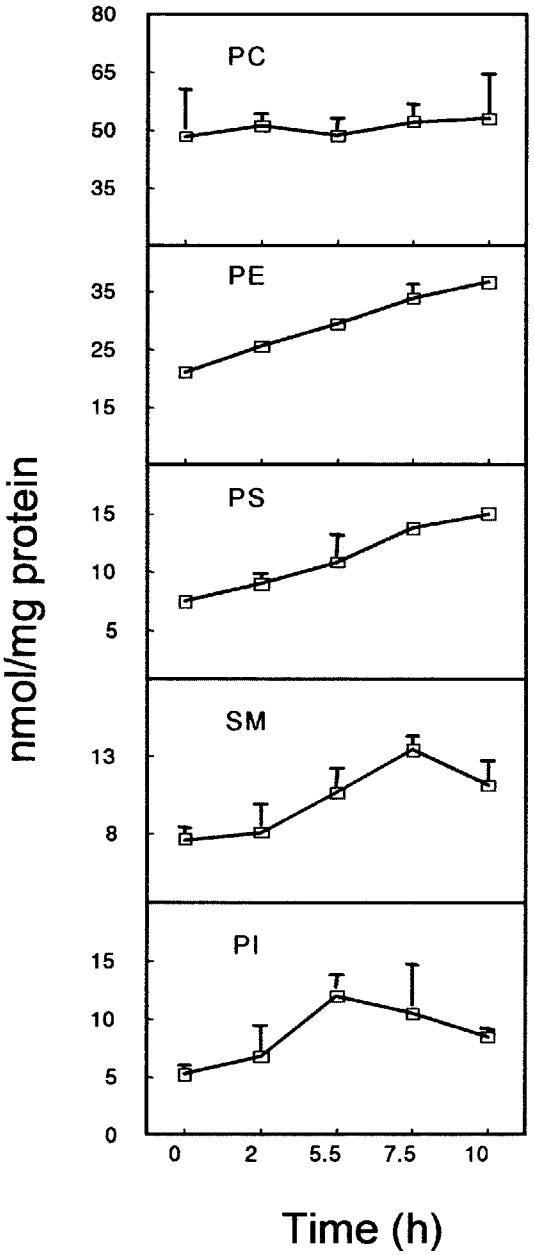

Figure 4 Time course of phospholipid mass changes in MDCK cells during the first 10 h of incubation in complete medium

For uniform labelling, MDCK cells were grown to confluence in the presence of 10 μCi/ml [^{32}P]P_i and then 6×10^6 cells were subcultured into an identical culture medium. At the indicated times, the cells were harvested, lipids were extracted and separated by TLC and total phospholipid phosphorus was determined. Results are expressed as nmol/mg of protein and are means ± S.D. of three independent experiments with incubations in duplicate.

125 ± 10 nmol/mg of protein after 10 h. Between 2 and 10 h, phospholipid content increased, with 11.9 % corresponding to PE, 5.9 % to PS, 2.9 % to SM, 1 % to PC and 0.7 % to PI. Figure 4 shows that there were transitory increments in PI and SM; PS and PE increased, whereas PC remained without any significant increment. It has been reported [15] that there is no increment in PC mass ([^{32}P]P_i steady-state labelling of PC) during the first 10 h of incubation in BAC1.2 F5 cells, due to PC turnover in the G_1 phase of growth. Although our cells were not synchronized, the absence of mass increment in PC suggested that the majority of

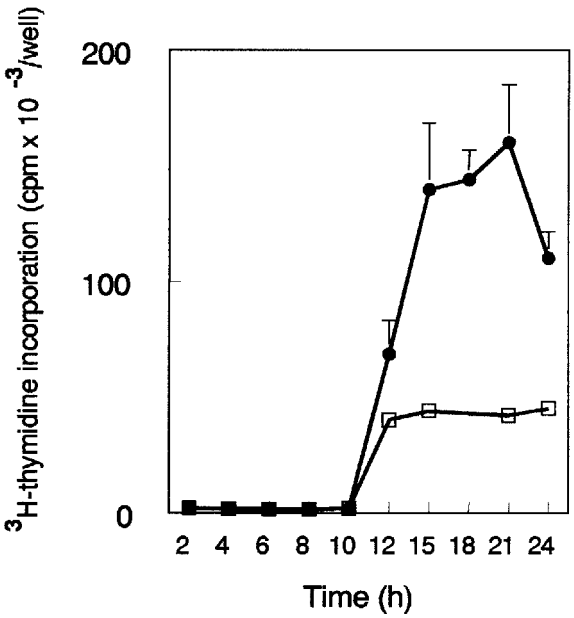

Figure 5 Inhibition of DNA synthesis by BCA

MDCK cells (1×10^5 cells/well) were seeded in complete DMEM in the absence (control, ●) or presence (□) of 2 mM BCA and 1 μCi/ml [^{3}H]thymidine. Incorporation of [^{3}H]thymidine was measured for 2 h interval from 2–24 h. Values are expressed as c.p.m. $\times 10^{-3}$/well and are means $\pm$ S.D. of six separate experiments.

the cells were in the G_1 phase during this period of time. Similar results were obtained in uniformly labelled cells with [^{3}H]palmitic acid (results not shown).

Effect of BCA on DNA synthesis

Since DNA synthesis takes place in the S phase of the cell cycle, we determined [^{3}H]thymidine incorporation. The cells were treated with a pulse of [^{3}H]thymidine and incorporation of the label into newly synthesized DNA was determined at approx. 2 h intervals from 2–24 h to identify the time of entry into S-phase. Figure 5 shows that, in response to FBS, cells DNA synthesis (enter S-phase) started to increase in cells 10 h after FBS stimulation and the peak incorporation occurred at 15–21 h. In cells that had been treated with FBS and BCA, the increase in [^{3}H]thymidine incorporation was decreased 3- to 4-fold between 15–21 h. FACS analysis showed that after 5.5 h of incubation 67–72 % of cells were in G_0/G_1, 10.3–15.3 % were in S phase and 17.8 % were in G_2M. After 16 h of incubation, 0–17 % of cells were in G_0/G_1, 68–82 % were in S phase and 15–18 % were in G_2M. After 16 h of incubation in the cells treated with BCA, 68 % of cells were still in G_0/G_1, 19 % were in S phase and 12 % were in G_2M, indicating the cells were arrested before or at the G_1 to S phase transition. Also, as shown by determining DNA synthesis, cells began to enter S phase 10–12 h after FBS stimulation, and DNA synthesis was inhibited by BCA.

DISCUSSION

In the present study, a clear correlation between SM synthesis, DAG generation and PKC activation was observed. BCA, an inhibitor of SPT, decreases SM synthesis, DAG generation and PKC activation. All of these processes are likely to take place in the early G_1 phase of the cell cycle (during the first 5.5 h of incubation).

During the first 5.5 h of incubation, 3.8 to 5.5 nmol of SM/mg of protein was synthesized (Figure 4) and the amount of DAG increased 49 ± 0.3 pmol/nmol of phospholipid (Table 2). Considering that there were approx. 100 nmol of phospholipid/mg of protein, the synthesis of SM was sufficient to account for all the DAG formed (4.9 nmol of DAG/mg of protein).

The observation that addition of D-erythrosphingosine to the BCA-treated cells restored the generation of DAG and a significant recovery of cell growth indicates that the inhibition caused by BCA was restored by metabolic complementation and therefore was specific. The contribution of SM synthesis to the generation of DAG was also shown when the synthesis of SM was inhibited at the level of ceramide synthase with fumonisin B1 and with D609 at the level of SM synthase. The inhibitors decrease SM synthesis and DAG generation to a similar extent. Although D609 may also inhibit PC-PLC, it is unlikely that fumonisin B1 inhibited PC-PLC. Propranolol has been reported [22] to inhibit PAP almost completely at a concentration of 100 μM, and we have found that the level of DAG was not decreased in MDCK cells treated with 250–500 μM propranolol (results not shown).

Considering all the above-mentioned results, it seems that PKC activation is a prerequisite for the transit of the MDCK cells through the cell cycle (G_1 to S phase) and that the DAG generated during SM synthesis is a necessary event for such activation. The pool of cellular DAG analysed in this study is derived from PC, not by the activation of catabolic pathways (PLC or PLD + PAP), but rather through the synthesis of SM.

There is abundant evidence that PKC controls the expression genes by altering the activity of certain transcription factors [1]. In some cell systems, activation of PKC with phorbol esters exerts positive or negative regulatory effects depending on the extent of PKC activation. In the early G_1 phase, PKC activation induces DNA synthesis, whereas activation in late G_1 phase inhibits DNA synthesis; thus, depending on the timing of PKC activation with phorbol esters during the G_1 phase, PKC may act at two independent points in early and late G_1 phase to regulate DNA synthesis, suggesting that PKC is a co-ordinator of the G_1/S transition [23]. We have found that inhibition of PKC (with 0.1 μM staurosporine, 25 μM H7 and 2 μM sphingosine) blocks head regeneration in hydra [24], and inhibition of growth of human tumour cell lines in nude mice by an antisense oligonucleotide inhibitor of PKCα expression has been reported [25].

Evidence for the involvement of SM synthesis in primary astrocyte proliferation arose from the finding that 10–20 μg/ml D609 decreased [^{3}H]thymidine incorporation by 40–50 % [26]. In MDCK cells, 140 nM fumonisin B1 or 20 μg/ml D609 decreased proliferation by 40 and 50 % respectively, whereas 1 μM fumonisin B1 induced a 90 % decrease in proliferation, and 40 μg/ml D609 caused complete inhibition.

PMA addition was capable of activating PKC in the BCA-treated cells; however, PMA was unable to restore proliferation, because the SM synthesis necessary for this process was inhibited by BCA. PKC activation was required, but no sufficient to restore proliferation.

The observations reported in the present study suggest that the sphingolipid and glycerophospholipid signalling may be coupled or interrelated with the opposing effects of ceramide and DAG. A perturbation of this ratio, whether by changes in ceramide, DAG or both, could therefore lead to changes in cell growth and viability. The complexity and interconnection of lipid metabolism has been reviewed (see [20,27,28]) and sphingosine has been reported to affect several pathways of phosphatidic acid metabolism, including its formation from activation of PLD, activation of the 80 kDa form of diglyceride kinase and inhibition of phosphatidic acid phosphohydrolase and CTP: phosphocholine

cytidylyltransferase. Different SM synthase activities have been described in various cellular compartments, such as Golgi, plasma membrane and mitochondria, but none of them has been purified/or cloned [27]. Some of the important questions to be elucidated included, how is the metabolism of SM regulated? Which are and where are the enzymes that participate in the synthesis, transformation and/or degradation of SM?

We thank Dr A. García-Sainz and Dr A. Zentella for critically reviewing the manuscript. This work was supported by a Consejo Nacional de Ciencia y Technología (CONACYT) Grant 211085-5-26404N and fellowship to R. C. L.-S.

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Received 5 November 2002/11 April 2003; accepted 14 April 2003
Published as BJ Immediate Publication 14 April 2003, DOI 10.1042/BJ20021732