The differential regulation of cyclic AMP by sphingomyelin-derived lipids and the modulation of sphingolipid-stimulated extracellular signal regulated kinase-2 in airway smooth muscle

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We report that sphingosine and short-chain ceramides activate adenylate cyclase and stimulate intracellular cyclic AMP formation in airway-smooth-muscle (ASM) cells. In each case, there is a conditional requirement for GTP- $G_s\alpha$. Sphingosine utilizes a protein kinase C-dependent pathway to elicit activation of adenylate cyclase, whereas for short-chain ceramides the mechanism remains unidentified. In contrast, sphingosine phosphate inhibits G_s -stimulated cyclic AMP formation via a G_i -dependent mechanism. Therefore, the potential interconversion of sphingosine and sphingosine phosphate is a switch that can elicit reciprocal changes in cyclic AMP levels. This may have a significant impact upon the regulation of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal specific kinase (JNK) by sphingolipids and may help to explain how growth factors that utilize these second messengers evoke pleiotropic responses such as proliferation and cell survival. In this context, short-chain ceramides are poor stimulators of ERKs in ASM

cells, and sphingosine is inactive, whereas both sphingolipids are powerful activators of the JNK module. Activated JNK catalyses N-terminal phosphorylation of c-Jun, a kinase cascade that programmes growth arrest. Therefore, in blocking ceramide-stimulated ERK-2 activity, cyclic AMP may allow the ceramide-dependent activation of JNK to programme cells to opt out of the cell cycle. In contrast, sphingosine phosphate activates ERK-2, potentiates growth-factor-stimulated DNA synthesis and fails to activate JNK, indicating that its sequential formation from ceramide and sphingosine may commit cells to DNA synthesis. ERK-2 can be activated by both cyclic AMP-sensitive c-Raf-1 kinase-dependent and cyclic AMP-insensitive c-Raf-1 kinase-independent pathways in ASM cells. In this context, sphingosine phosphate activates ERK-2 exclusively via c-Raf-1 kinase. Sphingosine phosphate-stimulated ERK-2 activity is also abolished by pertussis toxin, indicating that c-Raf-1 kinase is activated via a G_i -dependent mechanism.

INTRODUCTION

Agonist-dependent activation of sphingomyelinase elicits the formation of ceramide from plasma-membrane sphingomyelin [1,2]. For instance, ceramide is released as a consequence of tumour-necrosis-factor- α -stimulated sphingomyelin hydrolysis and can elicit both the autophosphorylation of a novel 97 kDa proline-directed protein kinase [3] and the activation of a specific PP2A-like phosphatase [4]. This novel kinase/phosphatase may perform an important role in NF- κ B activation and internucleosomal fragmentation of DNA (for a review, see [5]). Ceramide has also been demonstrated to inhibit lyso-phosphatidate-stimulated DNA synthesis in rat-1 fibroblasts [6] and can provoke the activation of mitogen-activated protein kinase (MAPK) [extracellular signal-regulated kinases (ERK) -1 and -2] in HL60 cells [7].

In Swiss 3T3 fibroblasts, ceramide is converted sequentially into sphingosine and sphingosine phosphate, the latter step being catalysed by a specific sphingosine kinase [8]. Sphingosine and sphingosine phosphate stimulate phospholipase D (PLD) activity and can promote cell proliferation [8–10]. Sphingosine also elicits the *in vitro* inhibition of phosphatidate phosphohydrolase and protein kinase C and the activation of diacylglycerol kinase

[11–13]. However, the relationship between these novel lipids, phosphatidate and mitogenic signal cascades, such as ERK, are not fully defined.

Of the signal components involved in the ERK signal cascade, c-Raf-1 kinase is believed to determine the flux through the pathway, this being governed by its ability to integrate multiple stimulatory intracellular signals, such as those arising from protein kinase C, G-protein $\beta\gamma$ subunits and p21^{ras} [14–17]. c-Raf-kinase has also been demonstrated to be an important target for signals that block activation of ERK. These include cyclic AMP which, in fibroblasts, acts via protein kinase A to catalyse phosphorylation of an N-terminal Ser-43 in c-Raf-1 kinase. This phosphorylation is purported to block the association of c-Raf-1 kinase with p21^{ras} [18–20]. Recent studies have demonstrated that sphingosine phosphate inhibits both forskolin- and isoproterenol-stimulated cyclic AMP formation in Swiss 3T3 fibroblasts [21], a response that is mediated by a pertussis-toxin-sensitive G-protein and that may involve a novel G-protein-linked receptor. However, there is no evidence that the products of sphingomyelin hydrolysis can stimulate cyclic AMP formation. This may, in part, be due to the complexity of ‘rules’ that determine whether adenylyl cyclase can integrate multiple signals. For instance, G_i -linked receptor agonists inhibit adenylate cyclase

Abbreviations used: ASM, airway smooth muscle; C16-ceramide, *N*-palmitoylsphingosine; C2-ceramide, *N*-acetylsphingosine; C6-ceramide, *N*-hexanoylsphingosine; DHS, donor horse serum; DME, Dulbecco’s modified Eagles medium; ERK-2, extracellular signal-regulated kinase-2; FCS, fetal-calf serum; HRP, horseradish peroxidase; JNK, c-Jun N-terminal-specific kinase; MAPK, mitogen-activated protein kinase; PDGF, platelet-derived growth factor; PMA, phorbol 12-myristate 13-acetate; PtdBut, phosphatidylbutanol; NP40, Nonidet P40; MEK, mitogen-activated ERK kinase; IBMX, 3-isobutyl-1-methylxanthine.

by utilizing $G_{i\alpha}$, although in the presence of coincidentally stimulated $G_{s\alpha}$, the corresponding $G_i \beta\gamma$ subunits can evoke marked stimulation of certain adenylate cyclase isoforms [22]. Furthermore, in airway-smooth-muscle (ASM) cells, phorbol 12-myristate 13-acetate (PMA) stimulates type 2 adenylate cyclase, although no measurable intracellular cyclic AMP response is apparent unless $G_{s\alpha}$ is coincidentally activated [23].

We have, therefore, assessed whether sphingomyelin-derived lipids can interact with the ERK-module in ASM cells. We conclude that only sphingosine phosphate elicits a substantial activation of c-Raf-1 kinase-dependent ERK-2, inhibits G_s -stimulated cyclic AMP formation and potentiates platelet-derived-growth-factor (PDGF)-stimulated DNA synthesis. When $G_{s\alpha}$ is activated, sphingosine and short-chain ceramides elicit cyclic AMP formation and this may represent a generalized signalling pathway that inhibits sphingolipid-dependent activation of the ERK module. This may be important in allowing concurrent flux through c-Jun N-terminal-specific kinase (JNK) to programme cells to opt out of the cell cycle, whereas the sequential formation of sphingosine phosphate from ceramide and sphingosine may commit cells to DNA synthesis.

MATERIALS AND METHODS

Materials

General chemicals, myelin basic protein, collagenase, elastase and soya-bean trypsin inhibitor were from Sigma Chemical Co. [^3H]Thymidine (5 Ci/mmol), [γ - ^{32}P]ATP (3000 Ci/mmol), [^3H]palmitate (50 Ci/mmol) and cyclic [^3H]AMP (24 Ci/mmol) were from Amersham International. All cell-culture media were from Gibco. Anti-ERK-1/2 antibody and *N*-acetyl-, *N*-hexanoyl- and *N*-palmitoyl-sphingosine (C2-, C6- and C16-ceramide), sphingosine and sphingosine phosphate were from Affiniti, Exeter, U.K.). C2-dihydroceramide was from Calbiochem. Recombinant mitogen-activated ERK kinase (MEK) and anti-c-Raf-1 kinase antibody were from Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). Reporter horseradish peroxidase (HRP)-anti mouse IgG was from the Scottish Antibody Production Unit (Carlisle, Scotland, U.K.). ECL (enhanced chemiluminescence) kits were from Amersham International. Male Dunkin-Hartley guinea-pigs (200–400 g) were used for isolation of tracheal smooth muscle.

Cell culture

The preparation of primary cultures of guinea-pig ASM cells was achieved as described previously [24]. The cells were maintained in Dulbecco's modified Eagle's medium (DME) containing 10% (v/v) foetal-calf serum (FCS) and 10% (v/v) donor horse serum (DHS) and were passaged twice, using trypsin, prior to experimentation. Cells were grown to confluence on 24- or six-well plates and routinely used between passage number 3 and 4 after the initial preparation. Their identity was confirmed to be smooth muscle by the presence of α -actin using a smooth-muscle-specific mouse anti α -actin monoclonal antibody [24].

PLD assays

Confluent ASM cells were preincubated with [^3H]palmitate (2 $\mu\text{Ci}/\text{ml}$) in DME containing 1% (v/v) FCS and 1% (v/v) DHS for 24 h. This procedure has previously been shown to result in the preferential incorporation of radioactivity into phosphatidylcholine, which contains 82% of the total lipid-associated radioactivity in comparison with only 6% associated with phosphatidylinositol, phosphatidylethanolamine, and phos-

phatidylserine combined [24]. PLD activity was determined using a transphosphatidyl transfer assay as described by us previously [24]. Briefly, cells were washed and preincubated in Krebs–Henseleit buffer before preincubation in the presence of 0.3% (v/v) butan-1-ol for 5 min prior to separate challenge with PMA, C2-, C6-ceramides, sphingosine and sphingosine phosphate for 10 min at 37 °C. A lipid extract of the cells was prepared and [^3H]phosphatidylbutanol ([^3H]PtdBut), which is a direct indicator of PLD activity [24], isolated by TLC using the upper phase of ethyl acetate/2,2,4-trimethylpentane (iso-octane)/water/acetic acid (11:5:10:2, by vol.). The R_f value of PtdBut, determined using an internal standard, was approx. 0.35.

Cyclic AMP measurements

ASM cells were placed in DME containing 1% (v/v) FCS and 1% (v/v) DHS for 24 h before use. Where indicated, cells were preincubated for 18 h with cholera toxin (0.5 ng/ml). Under these conditions only a small proportion of the total $G_{s\alpha}$ is activated [23]. The cells were washed twice in gassed (O_2/CO_2 , 19:1) Krebs–Henseleit buffer, pH 7.4, and preincubated in Krebs–Henseleit buffer with 1% (w/v) BSA for 30 min at 37 °C in air: CO_2 (19:1). The cells were then challenged with final concentrations of either bradykinin (100 nM), PMA (100 nM), C2 dihydro-, C2- and C6-ceramides, sphingosine and sphingosine phosphate (all at a concentration of 0.125–125 μM) or sphingomyelinase (0.1–10 units/ml) for indicated times at 37 °C. In some cases, cells were pretreated for 5 min with Ro318220 (10 μM), a selective protein kinase C inhibitor. We have previously demonstrated that Ro318220 blocks protein kinase C-stimulated cyclic AMP formation [25].

Incubations were terminated by the precipitation of the cells in 80% (v/v) ethanol. Samples were harvested and centrifuged at 14000 *g* and the cyclic AMP was extracted into ethanol, and then freeze-dried. Samples were taken for cyclic AMP determination using the [^3H]cyclic AMP binding assay with bovine heart cyclic AMP-binding protein that had been purified by us by the method of Rubin *et al.* [26].

[^3H]Thymidine incorporation

Cells (70–80% confluent) were placed in DME containing 0.1% (v/v) FCS and 0.1% (v/v) DHS for 24 h. After this time the cells were placed in DME with agonists at the indicated concentrations for a further 20 h. [^3H]Thymidine (0.25 $\mu\text{Ci}/0.25 \times 10^6$ cells) was added to incubations for a further 5 h. The incubations were terminated by washing each sample in 1 ml of ice-cold PBS, pH 7.4. The supernatant was aspirated and cells were washed three times (10 min each) with 1 ml of 10% (w/v) trichloroacetic acid. Nuclear material was dissolved in 0.25 ml of 0.1% (w/v) SDS/0.3 M NaOH and [^3H]thymidine incorporation quantified by liquid-scintillation counting.

Detection of ERK-2: shift assay

Cells were placed in DME containing 1% (v/v) FCS and 1% (v/v) DHS for 24 h. Cells were separately stimulated with PDGF (10 ng/ml), C2-, C16- and C6-ceramides, sphingosine and sphingosine phosphate (all at a final concentration of 100 μM). In some cases cells were preincubated for 18 h with either pertussis toxin (0.1 $\mu\text{g}/\text{ml}$) or cholera toxin (0.5 ng/ml). After treatment, for indicated times, the medium was removed and boiling sample buffer [0.125 M Tris/HCl (pH 6.7)/0.5 mM $\text{Na}_4\text{P}_2\text{O}_7/1.25$ mM EDTA/2.5% (v/v) glycerol/0.5% (w/v) SDS/25 mM dithiothreitol] was added and the cell lysates harvested. Samples were boiled for 5 min and then subjected to SDS/PAGE and trans-

ferred to nitrocellulose sheets. These were subsequently blocked in 5% gelatin in PBS, pH 6.8, at 37 °C for 1 h and then probed with an anti-ERK-1/2 antibody. Antibody was diluted 1:2000 (v/v) in 1% gelatin (w/v) and PBS plus 0.05% (v/v) Nonidet P40 (NP40). Nitrocellulose sheets were incubated with primary antibody at 37 °C for 12 h at 37 °C, prior to washing sequentially three times in PBS plus 0.05% (v/v) NP40. Detection of ERK-1/2 reactivity was achieved with a reporter HRP-linked anti-mouse antibody (1:2000 dilution, v/v) in 1% gelatin (w/v) and PBS plus 0.05% (v/v) NP40. Incubations were performed at 37 °C for 2 h. After washing the blots as described above, to remove excess reporter antibody, immunoreactive bands were detected with the ECL detection kit.

c-Raf-1 kinase immunoprecipitation

Cells were placed in DME containing 1% (v/v) FCS and 1% (v/v) DHS for 24 h. After stimulation with sphingosine phosphate (100 μ M), the medium was removed. Cells were lysed in ice-cold 1% Triton X-100/150 mM NaCl/10 mM Tris/HCl (pH 7.4)/1 mM EDTA/1 mM EGTA/0.5 mM Na_3VO_4 /20 mM NaF/0.2 mM PMSF/0.5% NP40 for 30 min. The material was harvested, centrifuged at 14000 g for 10 min at 4 °C and 200 μ l of cell lysate supernatant (equalized for protein) was taken and immunoprecipitated with specific antibodies {1 μ g of antibody, 500 μ l of water and 300 μ l of $\times 2$ immunoprecipitation buffer [1% Triton X-100/150 mM NaCl/10 mM Tris/HCl (pH 7.4)/1 mM-EDTA/1 mM EGTA/0.2 mM Na_3VO_4 /20 mM NaF/0.2 mM PMSF/0.5% NP40]}. After agitation for 1 h at 4 °C, 50 μ l of Protein A-Sepharose was added for 30 min at 4 °C. The subsequently obtained Protein A-Sepharose immune complex was collected by centrifugation at 14000 g for 5 min at 4 °C, washed three times in PBS plus 0.05% NP40 (v/v) and assayed for activity against purified recombinant MEK in the presence of [γ - 32 P]ATP. Kinase activity was assayed by incubation of the immunoprecipitates with 50 μ l of a 'phosphorylation cocktail' containing 1 μ g of MEK, 20 mM Hepes, pH 7.4, 5 mM MgCl_2 , 25 μ M ATP and 5 μ Ci of [γ - 32 P]ATP at 30 °C for 15 min. Incubations were terminated by the addition of an equal volume of boiling sample buffer. The resulting [32 P]phosphorylated MEK was resolved upon SDS/PAGE, gels were fixed and washed for 3–6 h in 5% (w/v) trichloroacetic acid and 1% $\text{Na}_4\text{P}_2\text{O}_7$ (w/v) and [32 P]phosphorylated MEK revealed by autoradiography. The [32 P]phosphorylated MEK band was quantified by densitometry.

RESULTS

Sphingolipids and cyclic AMP signalling

Recent studies in Swiss 3T3 fibroblasts have demonstrated that sphingosine phosphate inhibits both forskolin- and isoproterenol-stimulated cyclic AMP formation via a pertussis-toxin-sensitive G-protein [21]. In order to establish the wider significance of these findings, we have assessed the role of sphingosine phosphate in ASM cells. We have also separately measured the effect of short-chain ceramides and sphingosine, in order to establish whether the potential interconversion of these sphingolipids *in vivo* is likely to influence cyclic AMP signalling in mammalian cells. Consistent with studies by Spiegel and colleagues [21], sphingosine phosphate elicits a partial inhibition of cholera-toxin-stimulated cyclic AMP formation in ASM cells (Figure 1), whilst pertussis toxin (0.1 μ g/ml) blocks this reduction (results not shown). This indicates that sphingosine phosphate utilizes G_i , since ASM cells do not express the alternative pertussis-toxin substrate, G_o [27]. However, in cells treated with

Figure 1 Concentration-dependent effect of sphingosine phosphate (SphingosineP) on cyclic AMP (cAMP) in cholera-toxin-treated ASM cells

Concentration responses of sphingosine phosphate upon cyclic AMP formation in cholera-toxin-treated (0.5 ng/ml) ASM cells. Cyclic AMP values (means $\pm$ S.D., $n = 3$ separate cell preparations) are expressed as pmol/ 0.25×10^6 cells. The basal cyclic AMP level (in the absence of cholera toxin) was approx. 0.6 pmol/ 0.25×10^6 cells.

cholera toxin (0.5 ng/ml), short-chain (C2- and C6-) ceramides and sphingosine stimulate a concentration-dependent increase in cyclic AMP (Figures 2a–2c). This may play an important role in the regulation of cell proliferation, since cyclic AMP inhibits DNA synthesis and a maintained stimulation of cyclic AMP formation is expected to elicit growth arrest. However, a reduction in intracellular cyclic AMP in response to sphingosine phosphate is consistent with the ability of this sphingolipid to potentiate PDGF-stimulated DNA synthesis (Figure 3). These studies are important when considering the action of growth factors, such as PDGF, which stimulates sphingomyelin turnover and the acute activation of sphingosine kinase [8],

Figure 2 Concentration-dependent effect of C2-ceramide (C2), C6-ceramide (C6), sphingosine and sphingomyelinase (SMase) on cyclic AMP in cholera-toxin-treated ASM cells

Concentration responses for (a) C2-ceramide; (b) C6-ceramide; (c) sphingosine; (d) sphingomyelinase ($t = 10$ min stimulation) in cholera-toxin-treated (0.5 ng/ml) ASM cells. Cyclic AMP (cAMP) values (means $\pm$ S.D., $n = 3$ separate cell preparations) are expressed as pmol/ 0.25×10^6 cells. The basal cyclic AMP level (in the absence of cholera toxin) was approx. 0.6 pmol/ 0.25×10^6 cells. Abbreviation: U, units.

Figure 3 Concentration-dependence of sphingosine phosphate (SphingosineP)-stimulated potentiation of PDGF-stimulated DNA synthesis

The Figure shows DNA synthesis in response to various concentrations of sphingosine phosphate and a fixed concentration of PDGF (10 ng/ml) as measured by [3 H]thymidine incorporation. Results (means $\pm$ S.D.) for $n = 3$ separate cell preparations. ○, without PDGF; ●, with PDGF.

resulting in reciprocal changes in ceramide and sphingosine phosphate levels in intact cells [8].

These data suggest that the addition of sphingomyelinase to intact cholera-toxin-treated cells should generate sufficient quantities of ceramide and sphingosine to affect cyclic AMP formation, and this is indeed the case (Figure 2d). Importantly, the biologically inactive C2-dihydroceramide is completely ineffective at stimulating cyclic AMP formation [cholera-toxin-treated, basal, 1.75 ± 0.29 ; C2-dihydroceramide (125 μ M), 1.9 ± 0.5 (pmol/ 0.25×10^6 cells; $n = 3$ separate experiments)], indicating that the unsaturated C-C bond in the 4-5 position of ceramide is critical. Therefore, the specificity for unsaturated ceramide indicates that cyclic AMP formation is independent of non-specific lipid interactions.

Mechanisms regulating cyclic AMP signalling

If short-chain ceramides and sphingosine elicit an increase in cyclic AMP via the activation of adenylate cyclase, then the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) (100 μ M) will theoretically potentiate each cyclic AMP response. In Table 1 we demonstrate that this is the case. Equally, if short-chain ceramides and sphingosine inhibit cyclic AMP phosphodiesterase, they should mimic the phosphodiesterase inhibitor IBMX and potentiate forskolin-stimulated cyclic AMP formation. Indeed, this potentiation should exceed that induced by IBMX. This is based upon the observation that IBMX elicits an increase in intracellular cyclic AMP in cholera-toxin-treated cells that is smaller in magnitude to that elicited by either sphingosine or C6-ceramide (Table 1). However, whilst IBMX markedly potentiates forskolin-stimulated cyclic AMP formation, both C6-ceramide and sphingosine were ineffective (Table 1), indicating that they do not exhibit the characteristics of a phosphodiesterase inhibitor. Indeed, IBMX can still potentiate forskolin-stimulated cyclic AMP formation even in cells concurrently stimulated with C6-ceramide (Table 1). We therefore conclude that sphingosine and ceramide represent novel regulators of adenylate cyclase. This is supported by the observation demonstrating that the inhibition of adenylate cyclase by SQ22536 (100 μ M) blocks both sphingosine- and C6-ceramide-stimulated cyclic AMP formation (Table 2). In each case the stimulation of cyclic AMP formation

Table 1 Interaction of forskolin and IBMX with C6-ceramide and sphingosine-stimulated cyclic AMP formation

(a) Cholera-toxin-treated (0.5 ng/ml) ASM cells were pretreated with IBMX (100 μ M) (IBMX has a K_i of 1–5 μ M for ASM cyclic AMP phosphodiesterase activity) and then challenged with either C6-ceramide (125 μ M) or sphingosine (125 μ M) for 10 min. (b) ASM cells were treated with either C6-ceramide (125 μ M) or sphingosine (125 μ M) in the presence of forskolin (10 μ M) for 10 min. In some cases, cells were pretreated with IBMX (100 μ M) for 10 min. Cyclic AMP values (means $\pm$ S.D., $n = 3$ separate cell preparations) are expressed as pmol/ 0.25×10^6 cells. DMSO (1% final concn.) was included in the control and all the treatments.

(a)	
Agent	Cyclic AMP (pmol)
Control	3.23 ± 0.42
IBMX	5.47 ± 0.34
C6-ceramide	8.60 ± 0.6
Sphingosine	8.03 ± 0.9
IBMX/C6-ceramide	12.5 ± 0.5
IBMX/sphingosine	17.7 ± 1
(b)	
Agent	Cyclic AMP (pmol)
Control	0.2 ± 0.04
Forskolin	23.3 ± 0.85
Forskolin/IBMX	67.7 ± 6.9
Forskolin/Sphingosine	21.0 ± 2.5
Forskolin/C6-ceramide	23.0 ± 1.8
Forskolin/IBMX/C6-ceramide	68.7 ± 5.3

displays a conditional requirement for the coincident activation of $G_{s\alpha}$.

Ro318220, a potent protein kinase C inhibitor, attenuates sphingosine-stimulated cyclic AMP formation but fails to modulate the action of C6-ceramide (Table 3). We also demonstrate that Ro318220 inhibits PMA- and bradykinin-stimulated cyclic AMP formation (Table 3). We have previously proposed that bradykinin regulates the type 2 adenylate cyclase via a protein kinase C-dependent pathway that is downstream of the PLD-catalysed hydrolysis of phosphatidylcholine [23]. In this regard, it is significant that only sphingosine stimulates PLD activity in

Table 2 Effect of the adenylate cyclase inhibitor, SQ22536 on sphingolipid-stimulated cyclic AMP formation in cholera toxin-treated ASM cells

Cholera-toxin-treated (0.5 ng/ml) ASM cells were incubated in the absence or presence of SQ22536 (100 μ M) for 15 min prior to challenge for 10 min with either C6-ceramide (125 μ M) or sphingosine (125 μ M). Cyclic AMP values (mean $\pm$ S.D., $n = 3$ separate cell preparations) are expressed as pmol/ 0.25×10^6 cells. * $P < 0.01$ versus cyclic AMP response in the absence of SQ22536 (100 μ M). DMSO (1% final concn.) was included in the control and all the treatments.

Agent	Cyclic AMP (pmol)	
	Untreated	SQ22536
Control	3.8 ± 1.2	$0.6 \pm 0.3^*$
C6-ceramide	28 ± 2.8	$0.65 \pm 0.2^*$
Sphingosine	9.6 ± 0.4	$0.8 \pm 0.3^*$

Table 3 Effect of Ro318220 upon bradykinin-, PMA-, sphingosine- and C6-ceramide-stimulated cyclic AMP formation in cholera-toxin-treated ASM cells

The effect of bradykinin- (100 nM), PMA- (100 nM), C6-ceramide- (125 μ M) and sphingosine- (125 μ M) on cyclic AMP formation in cholera-toxin-treated (0.5 ng/ml) ASM cells in the presence and absence of Ro318220 (10 μ M). Cyclic AMP values are expressed as pmol/ 0.25×10^6 cells and are means $\pm$ S.D. for $n = 4$ separate cell preparations. * $P < 0.01$ versus cyclic AMP in absence of Ro318220. DMSO (1% final concn.) was included in the control and all the treatments.

Agent	Cyclic AMP (pmol)	
	Untreated	Ro318220
Control	2.8 ± 0.1	3.1 ± 0.2
Bradykinin	52 ± 10	$17.5 \pm 1.5^*$
PMA	21.5 ± 7.5	$1.7 \pm 0.5^*$
C6-ceramide	10 ± 4.1	8.4 ± 3.6
Sphingosine	7.2 ± 0.4	$4.1 \pm 1.15^*$

ASM cells (Table 4) and is therefore the only sphingolipid that can potentially activate protein kinase C via this route. In this context, it is also important to note that the type 2 adenylate cyclase is characterized by its ability to detect and integrate coincident G_{α} and protein kinase C signals [25].

The formation of cyclic AMP in response to short-chain ceramides and sphingosine are kinetically distinct. C6- and C2-ceramide stimulate cyclic AMP formation with sustained kinetics (Figure 4), whereas sphingosine elicits transient cyclic AMP formation, characterized by a rapid increase in the cyclic AMP signal which slowly declines to below basal levels after 45 min of cell stimulation (Figure 4). We cannot exclude the possibility that sphingosine is slowly converted into sphingosine phosphate under these conditions. As shown in Figure 1, sphingosine phosphate inhibits G_s -stimulated cyclic AMP formation and may account for the transient cyclic AMP response. In contrast, the direct addition of sphingosine phosphate to cells elicits an acute inhibition of cholera-toxin-stimulated cyclic AMP formation, followed by a delayed increase in cyclic AMP levels ($t < 10$ min), probably a consequence of its slow back-conversion into sphingosine (results not shown).

Table 4 Effect of PMA, sphingosine, sphingosine phosphate and C6-ceramide on PLD activity in ASM cells

The effect of dihydro-C2-ceramide (100 μ M), C6-ceramide (100 μ M), PMA (100 nM), sphingosine (100 μ M) and sphingosine phosphate (100 μ M) on [3 H]PtdBut formation in ASM cells. [3 H]PtdBut values are expressed as d.p.m./ 0.25×10^6 cells and are means $\pm$ S.D. for $n = 4$ separate cell preparations. DMSO (1% final concn.) was used as the vehicle for the sphingolipids. * $P < 0.001$ versus control, † $P < 0.001$ versus DMSO.

Agent	[3 H]PtdBut (d.p.m.)
Control	520 ± 17
DMSO (vehicle)	836 ± 24
PMA	$3392 \pm 164^*$
C6-ceramide	952 ± 111
Sphingosine	$1801 \pm 169^\dagger$
Sphingosine phosphate	890 ± 109
Dihydro-C2-ceramide	866 ± 19

Figure 4 Kinetics of C2-ceramide, C6-ceramide and sphingosine-stimulated cyclic AMP (cAMP) formation in cholera-toxin-treated ASM cells

Kinetics of control (○), C2-ceramide- (125 μ M, ▲), C6-ceramide- (125 μ M, ●) and sphingosine-stimulated (125 μ M, □) cyclic AMP formation in cholera-toxin-treated (0.5 ng/ml) ASM cells. Cyclic AMP values (means $\pm$ S.D., $n = 3$ separate cell preparations) are expressed as pmol/ 0.25×10^6 cells.

Effect of sphingolipids on ERK-2 activity: modulation by cholera and pertussis toxin

ERK-2 is expressed as a 42 kDa MAPK and its activation in response to both growth-factor and G-protein-linked-receptor stimulation is indicated by its retarded electrophoretic mobility upon SDS/PAGE: a consequence of its phosphorylation by its 'upstream' activating kinase, MEK. A maximal concentration of PDGF (10 ng/ml) can induce rapid activation of ERK-2, resulting in all of the immunodetected ERK-2 displaying retarded mobility on SDS/PAGE (Figure 5). In contrast, cell-permeable ceramides (C2- and C16-ceramide) elicit an activation of ERK-2 which results in only a fraction of the immunodetected ERK-2 displaying retarded mobility (Figure 5). Sphingosine is completely ineffective (Figure 5), although it does activate PLD activity in these cells (Table 4).

Sphingosine phosphate induces activation of at least 50% of the immunodetected ERK-2 (Figure 5), and we demonstrate here that it also activates c-Raf-1 kinase (Figure 6). We have previously shown that ERK-2 can be activated by both c-Raf-1 kinase-

Figure 5 Effect of sphingolipids and cholera toxin on ERK-2

The Figure shows a shift blot demonstrating the sphingolipid-induced activation of ERK-2 and the inhibitory effect of cholera-toxin pre-treatment (0.5 ng/ml). Lanes 1 and 4, non-challenged cells; lanes 2 and 5, C2-ceramide challenged (100 μ M); lanes 3 and 6, C16-ceramide-challenged (100 μ M). Lanes 1–3, control cells; lane 4–6, cholera-toxin-treated cells. Lanes 7 and 10, PDGF-challenged (10 ng/ml); lanes 8 and 11, sphingosine-challenged (100 μ M); lanes 9 and 12, sphingosine phosphate-challenged (100 μ M). Lanes 7–9, control cells; lanes 10–12, cholera-toxin-treated cells. This is a representative result of an experiment performed three times. ERK-1/2 and activated ERK-2 are denoted by arrows.

Figure 6 Sphingosine phosphate stimulates c-Raf-1 kinase activity in ASM cells

The Figure shows the effect of sphingosine phosphate (100 μ M) on c-Raf-1 kinase activity. Lane 1, unstimulated cells; lane 2, sphingosine phosphate-stimulated (100 μ M). This is a representative result of an experiment performed on four separate cell preparations. [32 P]MEK is denoted with an arrow.

Figure 7 Effect of pertussis toxin on sphingosine phosphate-induced activation of ERK-2

The Figure shows a shift blot demonstrating the effect of pertussis-toxin pre-treatment (0.1 μ g/ml) on sphingosine phosphate-induced activation of ERK-2. Lanes 1 and 4, non-challenged cells; lanes 2 and 3, sphingosine phosphate-challenged (100 μ M). Lanes 1 and 3, control cells; lanes 2 and 4, pertussis-toxin pre-treatment. This is a representative result of an experiment performed three times. ERK-2 and activated ERK-2 are denoted by arrows.

dependent and independent pathways in ASM cells (N. J. Pyne and S. Pyne, unpublished work). Only the c-Raf-1 kinase-dependent ERK-2 is inhibited by cyclic AMP. For this reason it is important to establish whether sphingosine phosphate can also activate ERK-2 via c-Raf-1 kinase-independent mechanism. In cholera-toxin-treated cells, the sphingosine phosphate-induced activation of ERK-2 is completely abolished (Figure 5), and this occurs despite the partial inhibition of cholera-toxin-stimulated cyclic AMP formation. Therefore, sphingosine phosphate activates ERK-2 via a c-Raf-1 kinase-dependent mechanism in these cells.

Pertussis toxin catalyses the ADP-ribosylation of the α subunits of G_i and G_o and in ASM cells inhibits sphingosine phosphate-stimulated ERK-2 activity (Figure 7). ASM cells do not express G_o [27], indicating that G_i probably couples to the ERK-2 module.

C2-ceramide-stimulated ERK-2 activity is also abolished by cholera toxin, an event that occurs concurrently with the amplification of cyclic AMP formation (Figures 2 and 5).

DISCUSSION

Sphingosine and short-chain ceramides stimulate cyclic AMP formation in ASM cells. Cyclic AMP formation displays a conditional requirement for GTP- G_{sz} and is mediated by a protein kinase C- and a novel-ceramide-dependent activation of adenylate cyclase. Therefore, the potential growth-factor-induced interconversion of sphingosine and sphingosine phosphate can result in reciprocal changes in cyclic AMP. In this regard, there is direct evidence that PDGF activates sphingosine kinase and

the formation of sphingosine phosphate in Swiss 3T3 fibroblasts [8]. This may have a significant impact upon DNA synthesis, since cyclic AMP is inhibitory. Therefore, a reduction in the level of cyclic AMP may potentiate growth-factor-stimulated DNA synthesis. In support of this, sphingosine phosphate enhances PDGF-stimulated DNA synthesis in ASM cells. In contrast, short-chain ceramides and sphingosine are powerful activators of the JNK module (results not shown). Activated JNK catalyses N-terminal phosphorylation of c-Jun and evokes growth arrest [28]. When G_{sz} is activated, sphingosine and short-chain ceramides elicit cyclic AMP formation, and this may represent a generalized signalling pathway that inhibits sphingolipid-dependent activation of the ERK module. This may be important in allowing concurrent flux through JNK to programme cells to opt out of the cell cycle. We do not know whether the relatively poor stimulation of ERKs in other cell systems is due to ceramide-dependent stimulation of cyclic AMP formation. However, if a relatively small pool of activated G_s is present in these cells, then conditional stimulation of cyclic AMP formation is possible.

Only one recently published article has demonstrated the stimulation of ERK-2 by sphingosine phosphate [29]. Whilst that study identified a role for a pertussis-toxin-sensitive G-protein, the authors were unable to discriminate whether either G_i or G_o is responsible for coupling to the ERK-2 module. Our study provides two novel aspects which are important advances. Firstly, in demonstrating that pertussis toxin blocks the activation of the ERK-2, we can establish that sphingosine phosphate utilizes G_i . Secondly, we demonstrate for the first time that sphingosine phosphate-stimulated ERK-2 activity is completely inhibited by a cyclic AMP-dependent mechanism and therefore only utilizes c-Raf-1 kinase. We have previously shown that growth factors activate ERK-2 via both c-Raf-1 kinase-dependent and -independent pathways in ASM cells (results not shown). Only the c-Raf-1 kinase-dependent ERK-2 module is inhibited by cyclic AMP.

The ability of sphingosine phosphate to stimulate ERK-2 activity via a G_i -dependent mechanism implicates a role for $G_i\beta\gamma$ subunits. The overexpression of $\beta\gamma$ subunits in Cos-7 cells constitutively activates ERK [15,16], whereas expression of a mutant constitutively active form of $G_i\alpha$ has no stimulatory effect. The $\beta\gamma$ subunit dimer can bind to target proteins via a pleckstrin homology domain [15], and a number of p21^{ras}-associated proteins contain this domain. The association of $\beta\gamma$ subunit with these proteins may stimulate GTP loading of p21^{ras}, thereby enabling c-Raf-1 kinase docking and localization in the plasma membrane. Furthermore, G-protein $\beta\gamma$ subunits can activate an unidentified kinase that catalyses the rapid tyrosyl phosphorylation of Shc and the consequential recruitment of a Grb-mSos complex and the GTP loading of p21^{ras} [30].

Sphingomyelin-derived lipids can also elicit rapid regulation of the hydrolysis of choline-containing phospholipids. Phosphatidate has also been demonstrated to prolong the lifetime of GTP-loaded p21^{ras} [31] via activation of a GTPase inhibiting protein and inhibition of a GTPase-activating protein (GIP/GAP; [31]). The prolonged activation of p21^{ras} is expected to sustain the stimulation of ERK-2 via a p21^{ras}/c-Raf-1 kinase pathway. However, phospholipase D-derived phosphatidate is not sufficient alone to stimulate ERK-2, since sphingosine can modulate PLD activity in the absence of ERK-2 activation.

We conclude that the interconversion of sphingolipids enables diverse signal pathways to be activated, all of which can contribute to pleiotropic cell responses.

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