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SPHINGOMYELIN METABOLITES AS SECOND MESSENGERS IN AIRWAY SMOOTH MUSCLE CELL PROLIFERATION

N. MALEKI DIZAJI, Pharm.D., M.Sc., A. GARJANI, Ph.D., AND S. PYNE,* Ph.D.

*From the Department of Pharmacology, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran and the *Department of Physiology and Pharmacology, University of Strathclyde, Glascow, U.K.*

ABSTRACT

Sphingolipid metabolism was examined in guinea-pig airway smooth muscle cells stimulated by platelet-derived growth factor (PDGF) and 4β-phorbol 12-myristate 13-acetate (PMA), as mitogens and bradykinin (BK) as non-mitogen. Stimulation of the cells by PMA and PDGF for 60 min. at 37°C induced the following changes in sphingolipid metabolites: in cells prelabeled with [^{3}H] palmitate, a 1.2 fold increase in radio-labeled sphingosine, a concomitant 20% decrease in radio-labeled ceramide and no significant change in sphingomyelin level. Stimulation of the cells by BK induced no changes in sphingolipid levels at any time tested. This study demonstrates the existence of a "sphingomyelin cycle" in airway smooth muscle cells. Such sphingolipid cycles may function in a signal transduction pathway and in cellular proliferation.

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INTRODUCTION

Among the lipid families found in eukaryotic cell membranes, there are three main groups; the glycerophospholipids, cholesterol, and the sphingolipids (SPLs). Originally thought to be solely structural molecules in the membrane bilayer, lipids are emerging as key players in the transduction of extracellular stimuli across the plasma membrane, resulting in the biological regulation of cell function, growth and differentiation. Until recently, neither cholesterol nor the SPLs were thought to have any signalling capacity. However, within the last few years SPLs have emerged as another class of signal-transducing lipids. Paralleling the cycle first discovered for glycerophospholipids, a cycle has been described for one of the SPLs, sphingomyelin (SM)[1] (Fig. 1).

A tantalizing link between sphingolipids and signal transduction surfaced recently when sphingosine was found to inhibit protein kinase C *in vitro* and to be an inhibitor of a variety of protein kinase C-dependent processes *in vivo*. Thus it has been suggested that sphingosine is an endogenous inhibitor of protein kinase C and represents the missing functional link to the pathology of gangliosidoses. However, several other reports suggest that sphingosine might have complex biological effects which are, at least in part, protein kinase C-independent.[2] It has been reported that low concentrations of sphingosine stimulate the proliferation of confluent and quiescent Swiss 3T3 fibroblasts and also potentiate the mitogenic responses of other growth factors

via a kinase C-independent pathway.[3] This raises the intriguing possibility that sphingosine and possibly other breakdown products of cellular sphingolipids may play an important role as positive modulators of cell growth acting in a fundamentally different, protein kinase C-independent pathway and that other target(s) of sphingosine action still remain to be uncovered.[2]

This renewed interest in sphingomyelin metabolism has resulted in the discovery that sphingomyelin may be rapidly synthesized and degraded in response to various cellular signals. This has led to interest in the biological properties of ceramide and the discovery of the novel sphingolipid ceramide l-phosphate. In addition to the potential biological effects of sphingosine, recent evidence suggests that these other derivatives of sphingomyelin may also play a pivotal role in signal transduction. Thus, sphingomyelin and its derivatives may constitute a new pathway through which information may be transmitted.[4]

The aim of this project was to determine whether sphingolipid metabolism was implicated in the proliferation of the airway smooth muscle cell. The approaches taken to answer this question were to determine whether mitogenic and non-mitogenic agonists had differential effects upon sphingolipid metabolism.

MATERIALS AND METHODS

Materials

[^{3}H] Palmitate (specific activity 40-60 Ci/mmol) and [^{3}H] Thymidine (specific activity 5 Ci/mmol) were purchased from Amersham International plc (Amersham, U.K.). Tissue culture reagents and plasticware were obtained from Gibco BRL (Paisley, U.K.) and ICN Flow (High Wycombe, U.K.). Bradykinin (BK) was purchased from Calbiochem (Nottingham, U.K.). TLC plates (LK6D) were obtained from Whatman (Maidstone, U.K.). Sphingomyelin, sphingosine, ceramide and other standards were purchased from the Sigma Chemical Co. (Poole, U.K.), Affiniti (Nottingham, U.K.) and Lipid Products (Redhill, U.K.). All other reagents were of the highest grade commercially available.

Cell culture

Guinea-pig tracheal smooth muscle cells were grown in monolayer cultures as previously described.[5] The cells were subcultured onto plastic 24-well clusterplates (Gibco) in Dulbecco's modified Eagle's medium (DMEM) containing 7.5% sodium bicarbonate and supplemented with 10% fetal calf serum (FCS), 10% donor horse serum (HS), 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 2 mM L-glutamine and maintained at 37°C in a humidified atmosphere of air supplemented with 5% CO_2.

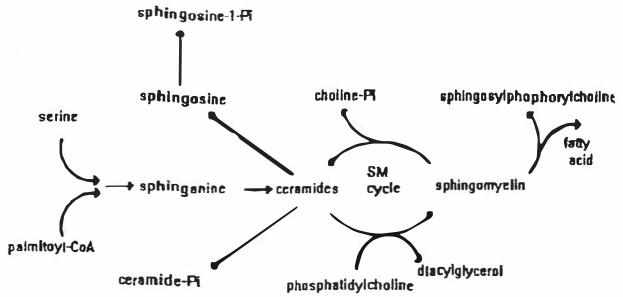

Fig. 1. Overview of sphingolipid metabolism.

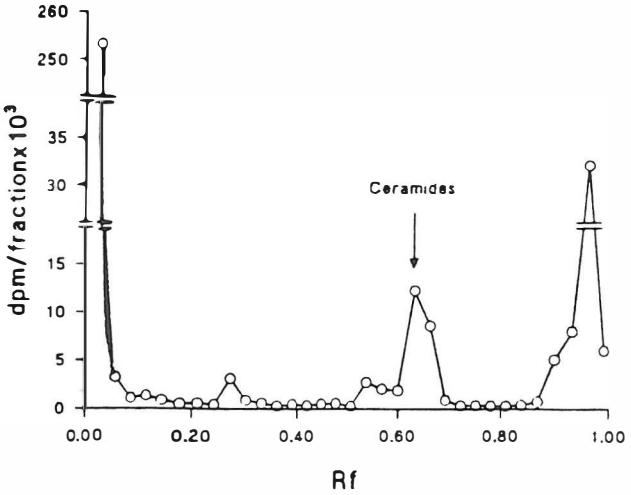

Fig. 2. A typical profile of radioactivity from [^{3}H] palmitate labeled airway smooth muscle cells using the ceramide TLC system (chloroform: methanol: acetic acid, 95: 5: 1 v/v). The mobility of ceramide is shown by the arrows.

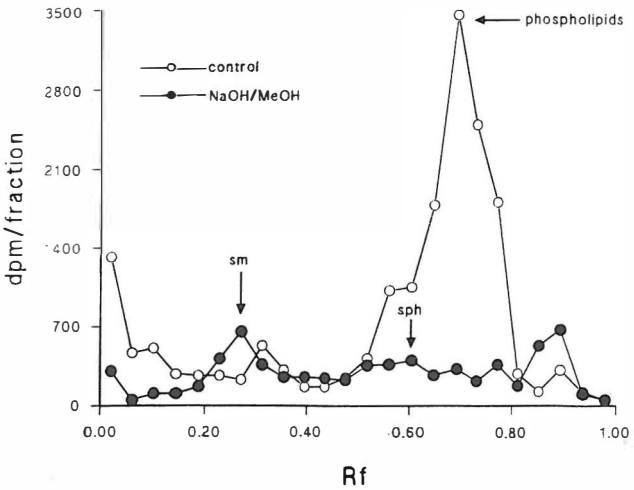

Fig. 3. A typical profile of radioactivity from [^{3}H] palmitate labeled airway smooth muscle cells before (open circles) and after (solid circles) treatment with NaOH/MeOH, using the sphingosine and sphingomyelin TLC system (butanol: water: acetic acid, 3: 1: 1 v/v). The mobility of sphingosine (Sph) and sphingomyelin (Sm) are shown by the arrows.

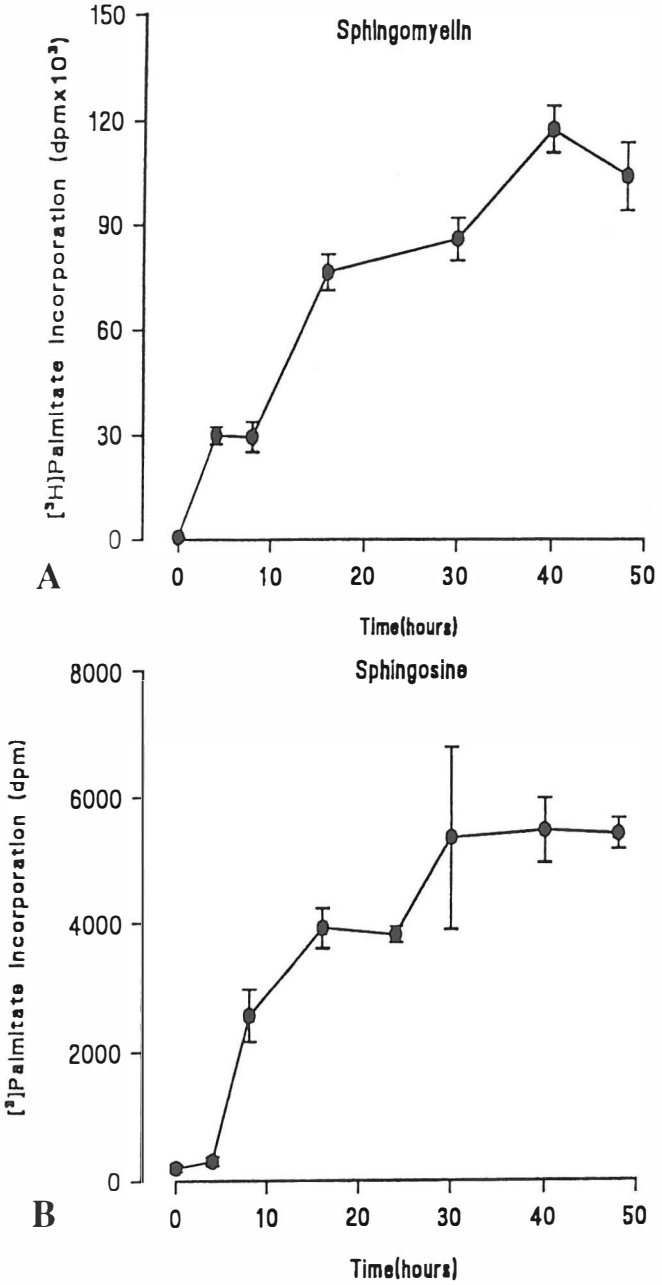

Fig. 4. Time course of incorporation of [³H] palmitate (2μCi/mL) into sphingomyelin (A) and sphingosine (B) in guinea-pig airway smooth muscle cells. Cells were incubated in DMEM/1% FCS/1% HS and 2 μCi [³H] palmitate per mL for the indicated time points. Lipid extracts were made, subjected to alkaline methanolysis and the sphingolipids resolved by TLC using butanol/acetic acid/water as described in the *Methods* section. Results are in dpm, mean±S.E.M., for combined data from three experiments.

Labeling and measurement of sphingolipids

To examine the effects of BK, PMA and PDGF on sphingolipid metabolism, airway smooth muscle cells were labelled with [³H] palmitate (2 μCi/mL) in DMEM containing 1% FCS and 1% HS for 48 h and washed with 500 μL Krebs solution. The cells were then incubated for 30 min with 250 μL of Krebs containing glucose (10 mM), calcium (1.5 mM), and bovine serum albumin (1g/100 mL). After 30 min, BK (100 nM, final), PMA (100 nM, final), PDGF-AB (10 ng/mL, final) or vehicle were added for the time indicated.

Incubations were terminated by removing the medium and the addition of 200 μL ice-cold acidified methanol (methanol: concentrated HCl, 200:1 v/v). Cells were scraped and transferred into glass vials. A second 200 μL of acidified methanol samples were left at room temperature for 20 min. to extract the lipids. The chloroform lipid extract was dried by centrifugal evaporation and redissolved in 300 μL chloroform: methanol (19:1, v/v). The samples were split in half; the first half was supplemented with unlabeled ceramide and applied to a TLC system for ceramide.[6] The plates were developed in chloroform: methanol: acetic acid (95:5:1, v/v). Radiolabeled ceramide that comigrated with the standard was scraped from the plate and quantified by liquid scintillation counting.

The second half of the samples was subjected to alkaline hydrolysis for 1 h,[7] in order to degrade the phospholipids, and then neutralized with 1 M HCl. After adding chloroform and water, the organic and aqueous phases were separated. The organic phase was supplemented with unlabeled sphingosine and sphingomyelin and applied to a TLC system for separation of sphingosine and sphingomyelin.[6] The plates were developed in butanol: acetic acid: water (3:1:1, v/v). Radiolabeled sphingosine and sphingomyelin that comigrated with standards were scraped from the plate and quantified by liquid scintillation counting.

Evaluation of TLC systems

To determine whether the TLC systems would provide optimal separation of sphingomyelin, sphingosine and ceramide from other sphingolipids and phospholipids, airway smooth muscle cells were labeled with [³H] palmitate and samples prepared as described above. Samples were supplemented with standards of ceramide (Cer), sphingosine (Sph), sphingomyelin (Sm), phosphatidylethanolamine (PtdEth), phosphatidylcholine (PtdCho), phosphatidylinositol (PtdIns), phosphatidylserine (PtdSer), phosphatidic acid (PtdOH), and diacylglycerol (DG) and applied either directly to the TLC system for ceramide or to the TLC system for Sph-Sm after alkaline hydrolysis, etc. Untreated lipid standards were run in parallel in both cases to enable identification of radio labeled compounds after their visualization using I_2. A profile of radioactivity from the sample lane was obtained by excising 0.5 cm fractions of silica and liquid scintillation counting.

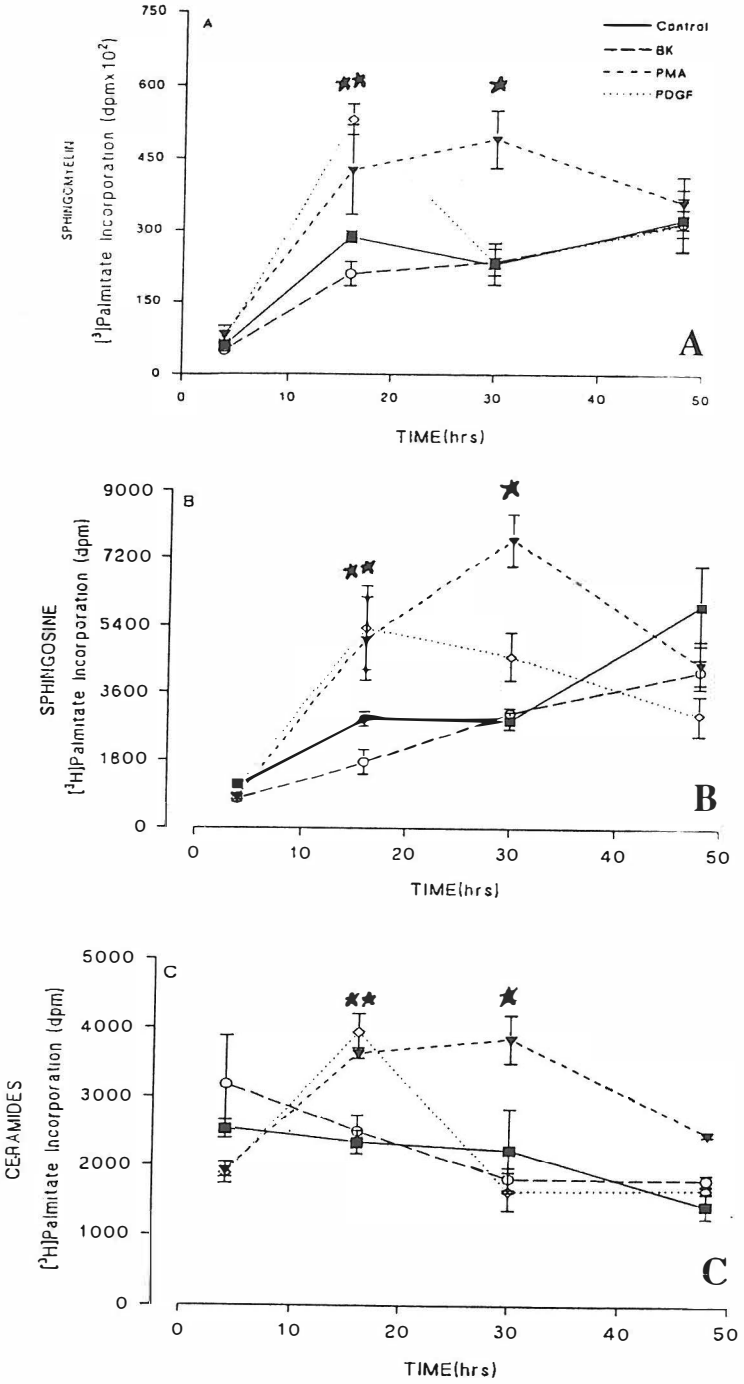

Fig. 5. Effect of agonists upon the time course of incorporation of [^{3}H] palmitate (2μCi/mL) into sphingomyelin (A), sphingosine (B) and ceramide (C) in cultured guinea-pig airway smooth muscle cells. Cells were incubated in DMEM/1% FCS/1% HS and 2μCi [^{3}H] palmitate per mL in absence, control, or presence of bradykinin (BK, 100nM), phorbol 12-myristate-13-acetate (PMA, 100 nM) and platelet-derived growth factor (PDGF-AB, 10 ng/mL). Lipid extracts were made as described in the *Methods* section. Results are presented in dpm, mean±S.E.M., for combined data from three experiments.

*p<0.05

**p<0.05

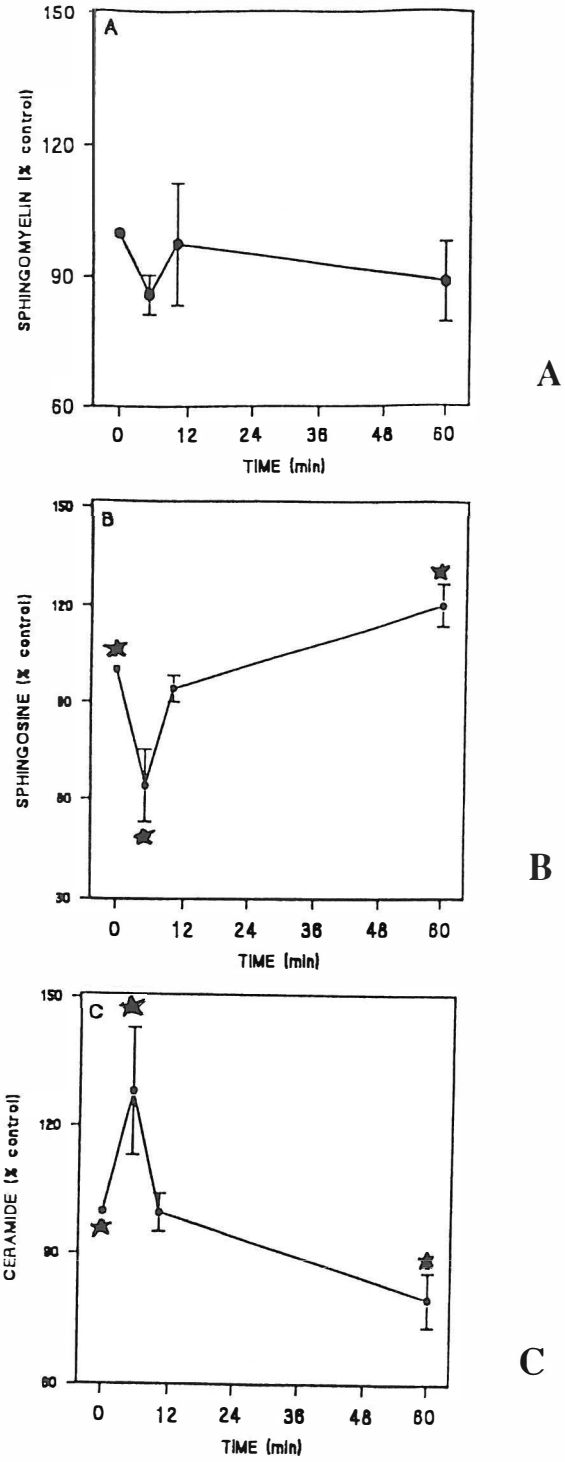

Fig. 6. Time course of the effect of platelet-derived growth factor (PDGF, 10 ng/mL) on the levels of sphingomyelin (A), sphingosine (B) and ceramides (C) in cultured guinea-pig airway smooth muscle cells. Data are presented as mean±S.E.M. of % of control values for each time point. The number of values are 12 from 4 independent experiments. The dpm values at zero time point were Sm (dpm= 65,272), Sph (dpm= 1,246) and Cer (dpm= 3,908).

*p<0.05, Mann-Whitney test

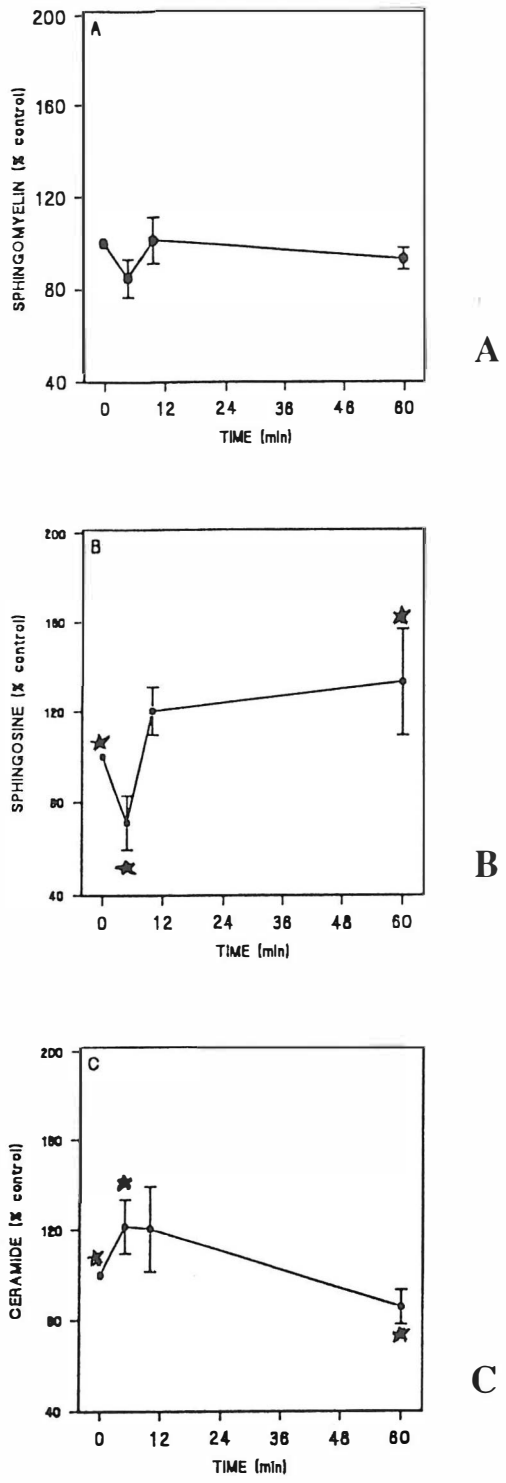

Fig. 7. Time course of the effect of phorbol 12-myristate 13-acetate (PMA, 100 nM) on the levels of sphingomyelin (A), sphingosine (B) and ceramides (C) in cultured guinea-pig airway smooth muscle cells. Data are presented as mean±S.E.M. of % of control values for each time point. The number of values are 12 from 4 independent experiments. The dpm values at zero time point were Sm (dpm= 65,272), Sph (dpm= 1,246) and Cer (dpm= 3,908).
*p<0.05, Mann-Whitney test.

Statistical calculations

Each assay described above was repeated 12 times, and mean±S.E.M. was calculated and analyzed using the Mann-Whitney test.

RESULTS

Ceramide profile

To examine whether the intracellular level of ceramide is regulated in response to different agonists, it was necessary to use a thin-layer chromatographic solvent system that would provide optimal separation of ceramide from other sphingolipids and major phospholipids. The graph in Fig. 2 illustrates a typical profile of radioactivity from [^{3}H] palmitate labeled cells using the ceramide TLC system. Rf (retardation factor) values in this TLC system, Sm (Rf= 0.00), Sph (Rf= 0.01), Cer (Rf= 0.62-0.67), Phospholipids (Rf= 0.00) and fatty acids (Rf= 0.95-1.00) indicate that ceramide is well resolved in this solvent system.

Sphingosine and sphingomyelin profile

Similarly, a thin-layer chromatographic solvent system that provides optimal separation of sphingosine and sphingomyelin from other sphingolipids and major phospholipids was used to enable agonist-stimulated changes in intracellular levels of sphingosine and sphingomyelin to be determined. The graph in Fig. 3 illustrates a typical profile of radioactivity from [^{3}H] palmitate labeled cells using sphingosine and sphingomyelin TLC system. Alkaline methanolysis degrades the phospholipids completely, as shown by the disappearance of the peak, but does not affect the sphingolipids. The Rf values of Sm, Sph and phospholipids before and after treatment indicate that Sm and Sph are readily resolved from glycerophospholipids by this method. The Rf values before treatment were: Sm (Rf= 0.2-0.35), Sph (Rf= 0.65), Cer (Rf= 0.83), phosphatidic acid (Rf= 0.64), phosphatidylcholine (Rf= 0.40), phosphatidyl ethanolamine (Rf= 0.59), phosphatidylserine (Rf= 0.54), and phosphatidylinositol (Rf= 0.53). After treatment the Rf values of degraded phospholipids were greater than 0.85 (Rf > 0.85), whereas the Rf values of sphingolipids were unchanged.

Time course of incorporation of [^{3}H] palmitate into sphingolipids

The kinetics of incorporation of [^{3}H] palmitate into sphingolipids was determined in order to identify the time at which a constant level of radioactivity in Sm (the precursor lipid) was attained. At this time, agonist-stimulated changes in radioactivity of Sm, Sph and Cer can be assumed to be representative of changes in the

chemical amounts of these lipids. It is shown that (Fig. 4) after about 40 h the radioactivity in sphingomyelin and sphingosine reaches a plateau. Thus in subsequent experiments, cells were labeled for 40-48 hr prior to stimulation.

Specific activation of sphingomyelin cycle by mitogens

Agonists which induce Sm hydrolysis and turnover would be expected to increase the kinetics of [^{3}H] palmitate into sphingolipids. Thus, to determine whether mitogens and non-mitogens have differential effects upon sphingomyelin hydrolysis and turnover, airway smooth muscle cells were incubated with [^{3}H] palmitate in the absence (control) or presence of BK (100 nM), PMA (100 nM) and PDGF (10 ng/mL). These concentrations of agonists have produced the maximal biochemical responses in airway smooth muscle cells.[7,8]

Bradykinin (BK), which is a non-mitogenic agonist, failed to increase sphingomyelin (Fig. 5A), sphingosine (Fig. 5B) and ceramide (Fig. 5C) levels over the same time interval with control ($p<0.05$). Phorbol 12-myristate 13-acetate (PMA), which activates protein kinase C, caused a significant increase ($p<0.05$) in sphingomyelin (Fig. 5A), sphingosine (Fig. 5B) and ceramide (Fig. 5C) levels, peaking at 30 h. These sphingolipids then returned to base-line levels (controls) by 48 h. Platelet-derived growth factor (PDGF) caused a significant increase in sphingomyelin (Fig. 5A), sphingosine (Fig. 5B) and ceramide (Fig. 5C) levels, peaking at 16 h ($p<0.05$). These sphingolipids then returned to base-line levels (controls) by 28-35 h.

These results would suggest that PMA and PDGF are both potent inducers of sphingomyelin turnover and that the sphingomyelin cycle may be implicated in the mitogenic effects of these agonists.

Effects of PDGF, PMA and BK on sphingomyelin, sphingosine and ceramide

To determine directly whether PDGF or PMA activate sphingolipid metabolism, airway smooth muscle cells were labeled with [^{3}H] palmitate for 48 h to allow equilibrium labeling of sphingolipid pools prior to stimulation. The time course of the PDGF and PMA effects are shown in Fig. 6 and Fig. 7, respectively. At earlier time points (5 min), both PDGF and PMA produced a statistically significant decrease ($p<0.05$) in the intracellular level of radiolabeled sphingosine (Figs. 6B, 7B) and concomitant increase in radiolabeled ceramide (Figs. 6C, 7C) relative to controls. Also at this time point there is a significant reduction in the intracellular level of sphingomyelin with both PDGF and PMA (Fig. 6A, 7A).

By increasing the time of stimulation, both PDGF and PMA produced a statistically significant increase ($p<0.05$) in radiolabeled sphingosine up to 12% (Figs. 6B, 7B) and a concomitant decrease ($p<0.05$) in radiolabeled ceramide, the immediate precursor of [^{3}H] sphingosine, down to 78-80% (Figs. 6C, 7C) in 60 min. relative to controls. There was no significant reduction in sphingomyelin levels in 60 min. relative to controls (Figs. 6A, 7A). This may be because only a small proportion of the large intracellular pool of Sm is hydrolysed to Cer and Sph. This may be estimated by comparing the dpm of Sph (dpm= 1,246) and Cer (dpm= 3,908) vs dpm of Sm (dpm= 65,272) at zero time point.

These flux measurements suggest that PDGF and PMA signalling in airway smooth muscle cells may be mediated by sphingolipid metabolism involving increased production of [^{3}H] sphingosine and a reduction in the levels of [^{3}H] ceramide in long term stimulation (60 min). In equilibrium labeling experiments, an increase in [^{3}H] sphingosine might arise predominantly by hydrolysis of [^{3}H] sphingomyelin and deacylation of the resulting [^{3}H] sphingosine.

This experiment was repeated for BK but sphingolipid levels were not significantly altered at any times tested (data not shown).

DISCUSSION

Numerous observations have suggested a connection between sphingolipid metabolism and cellular proliferation. The present study was designed to determine whether mitogens affect the level of intracellular sphingolipids in airway smooth muscle cells.

The identification of sphingomyelin turnover as a biochemical response to the action of PDGF and PMA, not BK, on airway smooth muscle cells is strongly presented in Fig. 5. Our findings in Fig. 6 and Fig. 7 suggest that Sph may be one of the endogenous factors that regulate PDGF and PMA effects in these cells. Sphingosine appears to have a central role in regulating some of the mitogen-induced biochemical changes that result in proliferation.

A model that links the observed PDGF-stimulated increase in sphingosine level to biochemical events associated with mitogenesis is suggested.[9] The PDGF-stimulated increase in [^{3}H] sphingosine (Fig. 6B) and concomitant decrease in [^{3}H] ceramide (Fig. 6C) are consistent with the hypothesis that binding of PDGF to its cell surface receptor stimulates conversion of sphingomyelin via ceramide to sphingosine and/or the conversion of ceramide to sphingosine. Alternatively, an increase in sphingosine could result from dehydrogenation of sphinganine[10] or deacylation of sphingomyelin to sphingosylphosphorylcholine and hydrolysis to sphingosine. However, it is unlikely that these pathways would result in the observed concomitant decrease in ceramide. Other mechanisms of formation of Cer/Sph

cannot be ruled out. The PDGF-stimulated increase in sphingosine could result from inhibition of sphingosine kinase. Furthermore, other sphingolipid metabolic pathways may be involved in PDGF signalling. Gangliosides, glycosphingolipids that are abundant in plasma membranes, may also modulate transmembrane signalling and regulate cellular proliferation.[11]

In conclusion, this study shows for the first time that mitogens influence sphingomyelin metabolism in airway smooth muscle cells. The sustained increase in the intracellular sphingosine level and concomitant decrease in the intracellular ceramide level may modulate the proliferative state of the cells, although the mechanism by which this is achieved remains to be determined.

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