N-Acetyl-sphingenine-1-phosphate is a potent calcium mobilizing agent

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Abstract Calcium mobilization induced by phosphorylated sphingoid bases was analyzed in calf pulmonary artery endothelial cells by confocal microscopy. A sphingenine-1-phosphate (SeP) analogue, *N*-acetyl-sphingenine-1-phosphate (N-C_2-SeP), exogenously added to these cells, caused a fast and transient intracellular rise in calcium and was as potent as SeP. A minimal concentration of 0.6 nM for N-C_2-SeP versus 1 nM for SeP was determined. The N-C_2-SeP-induced Ca^{2+}-signaling, like the response to SeP, was due to a release from thapsigargin-sensitive, ryanodine-insensitive, intracellular Ca^{2+}-stores and not to a Ca^{2+}-influx. N-C_2-SeP can be considered as a truncated ceramide-phosphate, a lipid already reported to be mitogenic (Gomez-Munoz, A., Duffy, P.A., Martin, A., O'Brien, L., Byun, H.S., Bittman, R. and Brindley, D.N. (1995) Mol. Pharmacol. 47, 833–839), an effect that might be secondary to Ca^{2+}-mobilization.

Key words: Ceramide-phosphate; Sphingosine-phosphate; Lysosphingolipid; Signal transduction; Calcium

1. Introduction

Phosphorylated sphingoid bases such as sphingenine-1-phosphate (SeP) are intermediates formed during sphingolipid breakdown [1]. In addition, these lipids are endowed with bioactivity and have been proposed to fulfill a second messenger role [2,3]. In 1990, Ghosh et al. [4] observed a sphingenine-induced Ca^{2+}-efflux from intracellular Ca^{2+}-stores in permeabilized DDT_1MF-2 smooth muscle cells, that was stimulated by ATP. This was a first indication that SeP – formed from sphingenine through the action of a sphingosine kinase – could be involved in Ca^{2+}-signaling. Subsequently, it was shown that externally applied SeP can induce Ca^{2+}-release in different cell types [2,3,5]. Intracellular calcium plays an important role in the regulation of several cellular functions ranging from secretion, contraction, phagocytosis, cell differentiation and proliferation to DNA synthesis and gene activation [6]. It has not been firmly established how SeP causes intracellular Ca^{2+}-release. Experimental evidence for different mechanisms can be put forward: an interaction with G-protein coupled plasma membrane receptors [5,7] followed by a phosphorylation/dephosphorylation cascade; a binding as a second messenger, after uptake or intracellular generation from sphingenine, to intracellular targets; a stimulation, partly modulated through G-proteins, of Ca^{2+}-entry as seen in thyroid FRTL-5 cells [8]; or a combination of these mechanisms. Data obtained in different cell types [9] indicate that SeP can act both extracellularly as a ligand and intracellularly as a second messenger.

In order to be considered as physiologically relevant, binding of SeP to its targets should be dependent on certain structural properties of the ligand. So far, due to difficulties to obtain and synthesize analogues of SeP, not much is known about structure-activity relationships involved in SeP-mediated events. Hitherto, the specificity of SeP has been compared mainly to that of other sphingolipids such as sphingenine and sphingenine-1-phosphocholine (lysosphingomyelin, SePC) [4,5,10–12]. These compounds and SeP are, however, metabolically interconvertible [1], complicating the interpretation of the experimental observations. To establish a more rigid structure-activity relationship, we started to analyze the Ca^{2+}-release in cultured calf pulmonary artery endothelial (CPAE) cells supplemented with different synthesized SeP analogues as reported in a preliminary form [13]. CPAE cells were chosen since they have been reported to be responsive to SeP [10] and the Ca^{2+}-signaling was monitored by confocal laser microscopy, a technique previously applied by one of us to investigate calcium signaling in retinal pigment endothelial cells [14]. Unexpectedly, the *N*-acetylated derivative of SeP turned out to be a potent calcium mobilizing lipid.

2. Materials and methods

2.1. Cell culture and fluorescence measurements

CPAE cells were obtained from the American Type Culture Collection (ATCC CCL 209, Rockville, MD, USA) and grown in Dulbecco's modified Eagle's medium (Gibco-BRL), supplemented with 20% (v/v) fetal calf serum. The culture medium was replaced every 2–3 days. Cells were plated in eight-chamber Lab-Tek chambered coverglass (Nunc) at a density of 5000 cells/cm^2 and analyzed one day after plating. Before the fluorescence analysis, cultures were incubated for 30 min at 37°C in 10 μM of fluo-3 acetoxymethyl ester (fluo-3 AM; Molecular Probes) dissolved in Hanks' balanced salt solution (HBSS; Gibco-BRL) containing 1.3 mM Ca^{2+}. Subsequently, each well was rinsed twice with HBSS, followed by addition of new HBSS and different amounts of SeP or SeP analogues, bound to albumin (molar lipid/albumin ratio = 1). The lipids were first dissolved in ethanol (2 mM) and diluted with 4 volumes of HBSS containing 0.4% (w/v) defatted bovine serum albumin, before further dilution in HBSS. Except for the addition of histamine (see further), each well was only tested once. Fluorescence was measured with the Meridian Insight confocal microscope (Meridian) based on an Olympus IMT2 inverted microscope with a D-plan APO100X (NA 1.25) or an S-plan APO 60X (NA 1.4) oil-immersion objective. Amplification and collection of the fluorescence signals and further image analysis was done as described in detail elsewhere [14]. Due to the lack of spectral shifts of

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Abbreviations: CPAE, calf pulmonary artery endothelial; fluo-3 AM, fluo-3 acetoxymethyl ester; IP_3, inositol-trisphosphate; HBSS, Hanks' buffered salt solution; N-C_2-SeP, *N*-acetyl-sphingenine-1-phosphate; SaP, sphinganine-1-phosphate; SeP, sphingenine-1-phosphate; SePC, sphingenine-1-phosphocholine

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fluo-3 fluorescence upon binding Ca^{2+}, measurements were normalized to the basal fluorescence intensities after background correction [14]. CPAE cultures were also exposed to other agonists known to modulate calcium to obtain information about the responsiveness of these cells and about the magnitude and time delay of their responses. [Arg^8]-vasopressin gave no or a very small Ca^{2+}-increase in these cells. Histamine, on the other hand, gave a rapid and clear response. Hence, histamine (10 μM final) was used as a control for cell viability and responsiveness in our experiments. In the different experiments performed, 60 to 90% of the cells present in a well were responsive to SeP. The refractory cells did also not react to histamine.

2.2. Synthesis of lipids

SePC (lysosphingomyelin), racemic at position 3, was purchased from Fluka Chemie (Buchs, Switzerland). Sphinganine-1-phosphate (SaP) and 3D,L-SeP (SeP) were prepared from sphinganine-1-phosphocholine and SePC, respectively, by phospholipase D treatment as described previously [15] and further purified by C_{18}-solid phase extraction [16]. *N*-acetyl-3D,L-sphingenine-1-phosphate (N-C_2-SeP) was obtained by acetylation of 3D,L-SeP with acetic anhydride [17]. Sphingolipid stocks were standardized by measurements of the amino groups with trinitro-benzenesulfonic acid [18] and/or phosphate groups, after wet ashing, by complexation with molybdate followed by reduction with ascorbic acid [19].

3. Results and discussion

In agreement with the data reported by Meyer zu Heringdorf et al. [10], treatment of CPAE cells with SeP resulted in intracellular Ca^{2+}-release. After addition of SeP in the low nM range to these cells, loaded with fluo-3 AM, an immediate and transient rise in the fluorescence was seen that returned to its control value after approximately 30 s (Fig. 1A; Table 1). Under our conditions, SeP concentrations as low as 1 nM were effective. This is comparable to the EC_{50} value of 0.8 nM reported by others for these cells [10] and of 2 nM for Hek293 cells [5].

The presence of a double bond seems quite important since SaP, a lipid identical to SeP except for the lack of the 4,5-*trans* double bond, was several-fold less active than SeP (Table 1). In our hands, SePC, tested up to 1 μM, did not cause an intracellular Ca^{2+}-increase. Others have reported an EC_{50} value of 260 nM for SePC in these cells [10]. Also the response of Hek293 cells to SePC is rather weak [5]. In DDT_1MF-2 smooth muscle cells [4], MC3T3-E1 osteoblasts [12], HL60 cells [20], and Swiss 3T3 cells [21] however, an instantaneous Ca^{2+}-release was seen after addition of SePC. Hence, the responsiveness to SePC, as to SeP, seems cell type dependent.

Rather unexpectedly, addition of *N*-acetylated SeP (N-C_2-SeP), an analogue that was synthesized to reveal the importance of the free amino group, resulted in a similar cellular response as that observed with SeP, i.e. a fast and transient change in fluorescence. A minimal concentration of 0.6 nM was determined for N-C_2-SeP (Table 1). The kinetic parameters for the responses to SeP and N-C_2-SeP were very similar (Table 1; Fig. 1A). Throughout the tested range of N-C_2-SeP, the magnitude of the individual cell response was rather similar (Fig. 1A, C).

To verify whether the N-C_2-SeP-induced intracellular Ca^{2+}-rise was due to a Ca^{2+}-influx, the response of CPAE cells was analyzed in Ca^{2+}-free medium. To exclude artefacts caused by a possible (plasma) membrane destabilization by N-C_2-SeP, these experiments were done at the maximal concentration of lipid tested. After a preincubation of 2 min in Ca^{2+}-free medium fortified with 2 mM EGTA, a Ca^{2+}-increase was still observed, and its magnitude appeared slightly larger than in

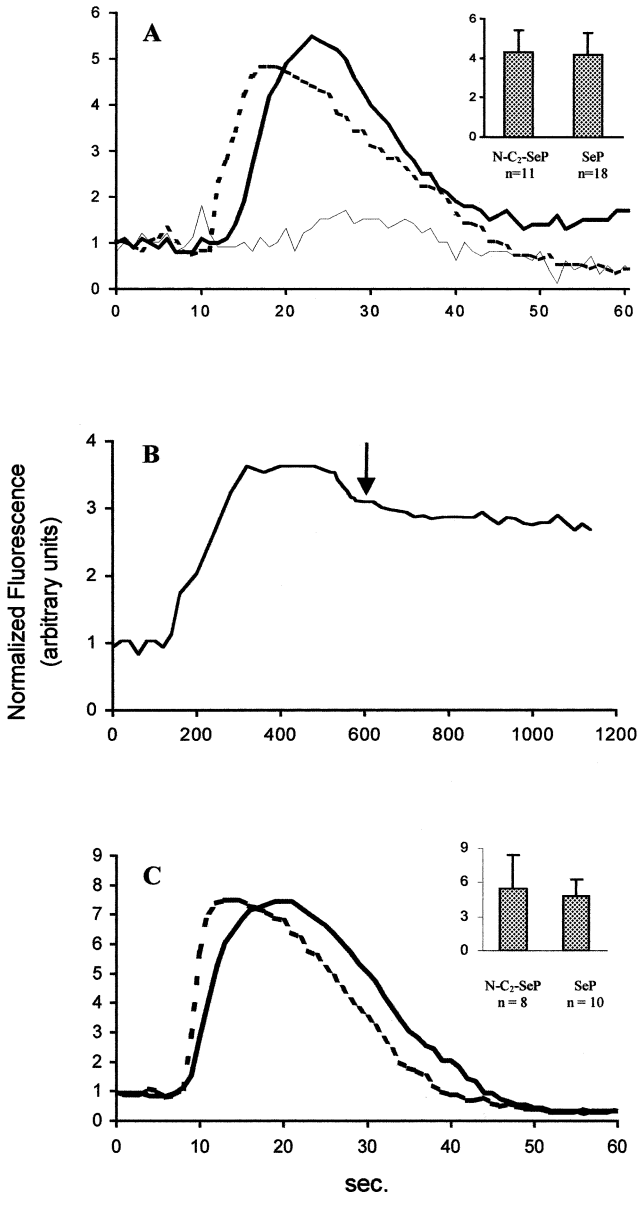

Fig. 1. Effect of N-C_2-SeP and SeP on Ca^{2+}-mobilization in CPAE cells. Panel A: CPAE cultures, loaded with fluo-3 AM, were stimulated at time zero with 1 nM of SeP (solid thick line), 0.6 nM of N-C_2-SeP (broken line) or medium without lipid (solid thin line). Panel B: Thapsigargin (1 μM) was added at time zero to fluo-3 AM loaded CPAE cultures to deplete the intracellular Ca^{2+}-stores. Subsequent addition of N-C_2-SeP or SeP (arrow) gave no longer an intracellular Ca^{2+}-rise. Panel C: Fluo-3 AM loaded CPAE cultures were preincubated for 15 min with ryanodine (10 μM) and stimulated at time zero with 100 nM SeP (thick line) or 100 nM N-C_2-SeP (broken line). The recordings in panels A–C represent the response monitored in one cell and normalized to its basal fluorescence as seen before addition of the lipid. The insets show the $[Ca^{2+}]_i$-rise, expressed in normalized fluorescence units (±S.D.) of *n* measurements, obtained in three separate experiments, after stimulation with N-C_2-SeP or SeP.

Ca^{2+}-containing medium at the maximal concentrations tested (Fig. 2). This suggests that the intracellular Ca^{2+}-increase was primarily due to a release from intracellular stores and not caused by a Ca^{2+}-influx or by membrane damage. Pretreatment of the cells with thapsigargin – a substance that depletes the intracellular Ca^{2+}-stores by inhibiting the Ca^{2+}-ATPase

Table 1
Ca^{2+}-changes in CPAE cells as revealed by normalized fluo-3 fluorescence monitoring

	Concentration (nM)	Normalized fluo-3 fluorescence	Delay time (s)	Time to peak (s)
SeP ($n=18$)	1	4.2 ± 1.1	9.7 ± 2.8	23.3 ± 6.5
SaP ($n=9$)	100	4.4 ± 1.6	13.5 ± 4.6	25.7 ± 3.7
N-C_2-SeP ($n=11$)	0.6	4.3 ± 1.1	10.4 ± 8.5	24.1 ± 11.3

CPAE cultures, loaded with fluo-3 AM, were stimulated with the indicated concentration of lipids. These concentrations are considered as minimal concentrations and were determined by testing the lipids, serially diluted and starting from 1 μM, until no response could be observed. Normalized fluorescence of n measurements is given in arbitrary units (± S.D.). Delay time is determined as the lag (in s ± S.D.) between addition of compound and onset of rise in fluorescence and time to peak as the period (in s ± S.D.) between addition of compound and maximal response.

pumps of the endoplasmic reticulum [22] – confirmed this observation. When CPAE cells were incubated with 1 μM thapsigargin in Ca^{2+}-containing medium, the fluorescence signals increased first and declined slowly afterwards. Subsequent addition of N-C_2-SeP or SeP gave no longer an intracellular Ca^{2+}-rise (Fig. 1B). Both the inositol-1,4,5-trisphosphate (IP_3) and ryanodine receptors are involved in the regulation of Ca^{2+}-release from intracellular Ca^{2+}-stores. To investigate whether the N-C_2-SeP-dependent Ca^{2+}-release occurred via ryanodine-sensitive Ca^{2+}-stores, cells were preincubated for 15 min with 10 μM ryanodine, followed by addition of the effective lipids. A normal response was seen (Fig. 1C), suggesting that the Ca^{2+}-release is caused by an IP_3-sensitive or another still unknown mechanism. For SeP, the latter possibility is more likely since heparin, an IP_3 antagonist, did not significantly alter the SeP-induced Ca^{2+}-release from microsomal vesicles prepared from DDT_1MF-2 cells [23].

As far as we are aware, N-C_2-SeP, a lipid that can be considered as a ceramide-phosphate analogue, has not been linked to Ca^{2+}-signaling. Interestingly, truncated ceramide-phosphates (*N*-acetyl- and *N*-octanoyl-SeP) were shown to be mitogenic for rat-1 fibroblasts [24] and perhaps this is caused by the Ca^{2+}-signaling we observed. Since long chain ceramide-phosphates do not appear to affect intracellular Ca^{2+}-release, at least in Hek293 cells [5], *N*-acetyl-SeP (and other truncated ceramide-phosphates) might be endowed with a specific bioactive role. Of course, it cannot be excluded that the non-responsiveness towards long chain ceramide-phosphates is due to solubility problems, or that the higher potency of N-C_2-SeP in CPAE cells is caused by its resemblance to lysophosphatidate or to platelet-activating factor, both bioactive compounds that bind to specific membrane receptors [25,26]. Whether N-C_2-SeP is present in biological samples has not been investigated, but theoretically it can be formed by acetylation of SeP, by phosphorylation of *N*-acetyl-sphingenine, or by hydrolysis of *N*-acetyl-SePC. The *N*-acetylation of sphingenine has recently been described [27]. Interestingly, the transferase involved uses platelet-activating factor as acetyl-donor, but the action of this enzyme on SeP was not investigated. The phosphorylation of *N*-long chain acyl-sphingenine (ceramide) was reported in HL60 cells [28] and in human neutrophils [29]. It has also not been established at which cellular site N-C_2-SeP (and SeP) is interacting, either at the plasma membrane or at the endoplasmic reticulum. During the course of our experiments, different approaches have provided evidence for the existence of multiple plasma membrane lysophospholipid receptors. van Koppen and coworkers showed that the response of a number of differentiated cell types to SeP and SePC and other lysosphingolipids varies considerably and postulates the presence of three types of receptors that interact with SeP and/or other lysosphingolipids [3,5]. By resemblance to the lysophosphatidate receptor, a number of other receptors that display specificity towards SeP such as edg1 [30], edg3 [31], and H218 [31] was recently cloned. Obviously, these are likely candidates for binding *N*-acetyl-SeP as well. On the other hand, Mao et al. [32] discovered a SePC-gated Ca^{2+}-channel, likely localized to the endoplasmic reticulum. Not much is known about the uptake and membrane translocation of phosphorylated sphingoid bases. Studies with labeled SeP [33] or SaP [34] indicate that these compounds, despite their charges, can cross the plasma membrane. The higher potency of N-C_2-SeP, lacking the positive charge, could therefore be as well caused by a better uptake, followed by binding, with or without a deacylation step, to its intracellular target.

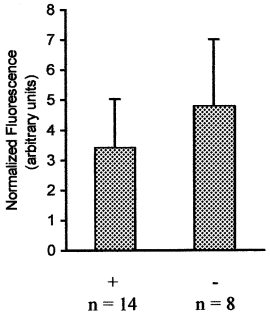

Fig. 2. Role of extracellular Ca^{2+} during Ca^{2+}-mobilization by truncated ceramide-phosphate. The bar graphs represent the $[Ca^{2+}]_i$-rise, expressed in normalized fluorescence units (± S.D.) of n measurements, observed in fluo-3 AM loaded CPAE cultures, after stimulation with 1 μM N-C_2-SeP in Ca^{2+}-containing (+) and in Ca^{2+}-free (−) medium.

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