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Sphingosine-1-phosphate stimulates cortisol secretion

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Abstract **We show here for the first time that sphingosine-1-phosphate (Sph-1-P) stimulates cortisol secretion in zona fasciculata cells of bovine adrenal glands. This effect was dependent upon protein kinase C (PKC) and extracellular Ca^{2+}, and was inhibited by pertussis toxin. Sph-1-P activated phospholipase D (PLD) through a pertussis toxin-sensitive mechanism, also involving extracellular Ca^{2+} and PKC. Primary alcohols, which attenuate formation of phosphatidic acid (the product of PLD), and cell-permeable ceramides, which inhibit PLD, blocked Sph-1-P-induced cortisol secretion. In conclusion, Sph-1-P stimulates cortisol secretion through a mechanism involving Gi/o protein-coupled receptors, extracellular Ca^{2+}, PKC and PLD.**

Key words: Sphingosine-1-phosphate; Ceramide; Cortisol secretion; Phospholipase D; Protein kinase C; Adrenal gland

1. Introduction

Sphingosine-1-phosphate (Sph-1-P) has been described as a potent bioactive lysophospholipid that is implicated in the regulation of many critical cell functions [1,2]. It is formed by ATP-dependent phosphorylation of sphingosine by sphingosine kinase, and can be metabolized by pyridoxal phosphate-dependent lyase and phosphatase activities [1,3]. Activation of sphingosine kinase and enhanced formation of Sph-1-P was shown to be induced by platelet-derived growth factor, but not epidermal growth factor, leading to mitogen-activated protein kinase activation and the regulation of cell proliferation [4]. In addition, Sph-1-P has been shown to act intracellularly as a second messenger to inhibit apoptosis and promote cell survival [1,2]. Another mechanism whereby Sph-1-P can elicit biological responses is its interaction with specific G protein-coupled receptors of the EDG (endothelin differentiation gene) family [1,5]. Sph-1-P is stored at high concentrations in human platelets, or in mast cells, and can be released into the blood stream upon activation by physiological stimuli [6]. Stimulation of these receptors has been associated with the regulation of cell differentiation, smooth muscle contraction, cell migration, inhibition of tumor cell invasion, and angiogenesis [5,7]. The biological effects elicited by Sph-1-P involve activation of diverse signaling pathways including stimulation of phosphatidylinositol-dependent phospholipase C (PI-PLC), mitogen-activated protein kinase ERK1/2, phosphatidylinositol 3-kinase, phospholipase D (PLD), or transcription factors such as AP-1 [1,5,8]. Furthermore, Sph-1-P can mobilize inositol trisphosphate (IP_3)-sensitive and -insensitive intracellular Ca^{2+} stores [9,10].

In the present study, we demonstrate for the first time that Sph-1-P stimulates the secretion of cortisol in zona fasciculata (ZF) cells of bovine adrenal glands and provide evidence on the mechanisms whereby Sph-1-P exerts this action. The data presented demonstrate a novel role of Sph-1-P in the regulation of steroid secretion.

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA; fraction V), and collagenase P and A were from Boehringer Mannheim (Mannheim, Germany). Angiotensin II, cortisol, Dulbecco's modified Eagle's medium (DMEM), EGTA, HEPES, pertussis toxin (PTX), lysophosphatidate (LPA), and sphingomyelinase (from *Bacillus cereus* or *Streptomyces* sp.) were from Sigma (St. Louis, MO, USA). [^{3}H]Myristate, [^{3}H]cortisol and *myo*-[1-2-^{3}H]inositol were supplied by American Radiolabeled Chemicals (St. Louis, MO, USA). Sphingosine, Sph-1-P, *N*-acetylsphingosine (C_2-ceramide), dihydro-C_2-ceramide, *N*-octanoyl-Sph-1-P, and phosphatidylethanol standard were from Avanti Polar Lipids (Alabaster, AL, USA). Fura 2-AM was from Molecular Probes (Eugene, OR, USA). 4β-Phorbol 12-myristate 13-acetate (PMA) was from Alexis (Läufelfingen, Switzerland).

2.2. Cell preparation and culture

Cells were isolated and purified from the ZF of adrenal glands obtained from 1 year old steers, as described [11]. Cells were seeded in 35 mm culture dishes (8×10^5 cells per dish), or in 12 well plates (3.5×10^5 cells per well). After 24 h, the medium was replaced by fresh DMEM supplemented with 10% fetal bovine serum (FBS) and cells incubated further for 2 days in a gassed, humidified incubator (5% CO_2 at 37°C) before use in experiments. This time was chosen because steroid output from these cells increases to a maximum by 48–72 h [11].

2.3. Determination of cortisol secretion

Cortisol output was measured by radioimmunoassay, as described [12]. Cells were washed twice in DMEM supplemented with 0.2% BSA, and incubated for 2.5 h in this same medium. No intermediate washes were carried out during the procedure to prevent the burst of sphingolipids and diacylglycerol that occurs rapidly after changing the medium [13,14]. Agonists were then added and cells incubated further for 2 h. The medium was then recovered for determination of cortisol content.

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Abbreviations: BSA, bovine serum albumin; C_2-ceramide, *N*-acetylsphingosine; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; IP_3, inositol trisphosphate; LPA, lysophosphatidate; PA, phosphatidate; PKC, protein kinase C; PLD, phospholipase D; PI-PLC, phosphatidylinositol-dependent phospholipase C; PMA, 4β-phorbol 12-myristate 13-acetate; PTX, pertussis toxin; Sph-1-P, sphingosine-1-phosphate; ZF, zona fasciculata

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2.4. Assay of PLD

PLD was determined by measuring the production of [^{3}H]phosphatidylethanol, as described [15]. Briefly, cells were incubated for 3 h with 1 μCi [^{3}H]myristate/ml to label cell phosphatidylcholine, washed twice and then incubated for 2.5 h with no intermediate washes. Ethanol, at a final concentration of 1%, was added 5 min prior to addition of agonists. Lipids were extracted as described [16] and separated by thin layer chromatography. The plates were developed with chloroform/methanol/acetic acid (9:1:1, v/v), and the position of lipids was identified after staining with I_2 vapor by comparison with authentic standards. Radioactive lipids were quantitated by liquid scintillation counting.

2.5. Assay of PI-PLC

PI-PLC was determined as described [17]. Briefly, ZF cells were incubated for 24 h at 37°C in DMEM supplemented with 10% FBS containing 1 μCi/ml of *myo*-[1-2-^{3}H]inositol to label cell phosphoinositides. Cells were then washed twice and incubated in BSA-free medium with 10 mM LiCl for 2.5 h. Agonists were added as required, and reactions stopped with 0.5 ml of 5% $HClO_4$ and 100 μl BSA (20 mg/ml). The [^{3}H]inositol phosphates were separated from [^{3}H]inositol by retention on columns of ion-exchange resin (Dowex AG1-X8, 100–200 mesh) as described [18].

2.6. cAMP determination

Cells were preincubated for 20 min with 1 mM isobutylmethylxanthine, prior to agonist addition, and then treated as described [19]. cAMP levels were determined using an enzyme immunoassay kit from Amersham.

2.7. Statistical analysis

Unless stated otherwise, results are expressed as means ± S.E.M. of the indicated number of experiments performed in triplicate. Statistical significance of the difference between means of control and experimental conditions was assessed with Student's paired *t*-test. Values of $P \leq 0.05$ were considered significant.

3. Results and discussion

Sph-1-P stimulated the secretion of cortisol in ZF cells of bovine adrenal glands. This effect was specific for Sph-1-P as other bioactive sphingolipids such as sphingosine, *N*-octanoyl-Sph-1-P, or the structurally related lysoglycerophospholipid LPA were without effect (Fig. 1). The cells were also stimulated with concentrations of these compounds of up to 50 μM, or with the cell-permeable C_2-ceramide (at 10 or 50 μM), but no elevation in cortisol levels was detected.

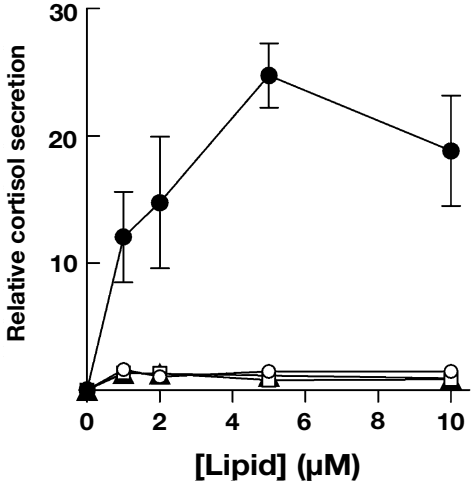

Fig. 1. Stimulation of cortisol secretion by different lipid agonists. Cells were stimulated for 2 h with increasing concentrations of Sph-1-P (solid circles), sphingosine (empty circles), *N*-octanoyl-Sph-1-P (squares), or LPA (triangles). For experimental details see Section 2. Results are expressed as fold stimulation relative to incubations with vehicle, and show the mean ± S.E.M. of four independent experiments, except for the value at 5 μM Sph-1-P, which is the mean ± S.E.M. of 36 experiments. Basal value of cortisol secretion was 7.6 ± 1.0 ng/mg protein (mean ± S.E.M., $n = 48$).

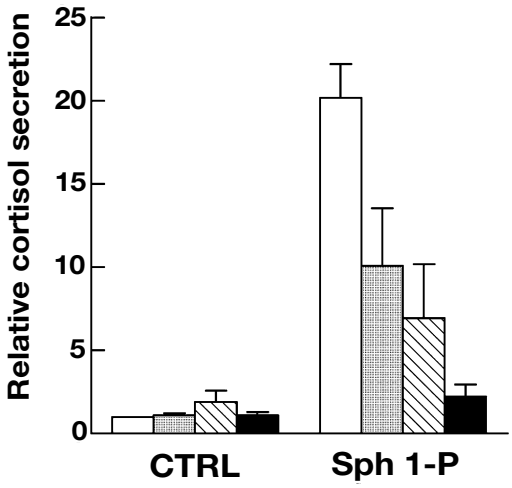

Fig. 2. Effect of PTX and PMA on Sph-1-P-stimulated cortisol secretion. Cells were treated as in Fig. 1. They were preincubated with vehicle (empty bars), 2 μM PMA for 48 h (hatched bars), 1 μg/ml PTX for 16 h (dotted bars), or with PTX and PMA (solid bars), as indicated. Sph-1-P (5 μM) was then added and the incubations continued for a further 2 h. CTRL indicates incubations in the absence of Sph-1-P (controls). Results are calculated as in Fig. 1, and they are expressed as mean ± S.E.M. of three independent experiments.

It is well established that adrenocortical ZF cells secrete high amounts of cortisol in response to ACTH, or β_1-adrenergic agonists, through stimulation of adenylyl cyclase [11,20]. Interestingly, Sph-1-P stimulated cortisol secretion to a similar extent to that of adrenaline, but in contrast to this hormone, it did not stimulate cAMP formation. In addition, Sph-1-P failed to alter ACTH-stimulated cAMP generation (data not shown). Although Sph-1-P is rapidly taken up by cells and acts intracellularly, some of its biological effects are caused by binding to specific receptors that are coupled to PTX-sensitive Gi/o proteins [1,21]. We found that the stimulation of cortisol secretion by Sph-1-P was potently decreased by PTX, suggesting that this is a process mediated by Gi/o-coupled receptors (Fig. 2). It was also reported that cortisol secretion can be regulated by protein kinase C (PKC) and Ca^{2+} independently of cAMP [22]. Therefore, we examined the implication of these factors in the stimulation of cortisol secretion by Sph-1-P. The involvement of PKC was studied by preincubating the cells with PMA (2 μM) for 48 h, as this condition causes downregulation of PKC in bovine adrenal glomerulosa cells [23]. Under these conditions, the cells lost their sensitivity to stimulation of cortisol secretion by PMA [cortisol output was decreased from 3.15 ± 0.60 fold to 1.12 ± 0.13 (mean ± S.E.M. of five independent experiments, $P < 0.05$)], and the effect of Sph-1-P was substantially decreased (Fig. 2). Similar results were obtained by preincubating the cells for 30 min with the selective PKC inhibitor Ro-32-0432 (1 μM) prior to Sph-1-P addition (not shown). Therefore, these results suggest that PKC is involved in Sph-1-P-stimulated cortisol secretion. This effect was almost completely abolished by combined preincubation with PMA and PTX (Fig. 2). We then evaluated

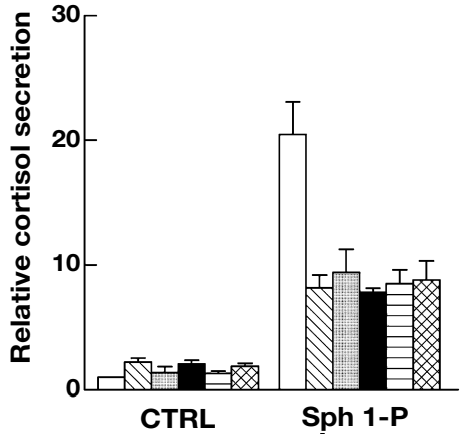

Fig. 3. Effect of EGTA, Fura 2-AM, and BAPTA-AM on Sph-1-P-induced cortisol secretion. Cells were preincubated for 30 min with vehicle (empty bars), 5 mM EGTA (hatched bars), 10 μM Fura 2-AM (dotted bars), 5 μM BAPTA-AM (striped bars), EGTA and Fura 2-AM (solid bars), or EGTA and BAPTA-AM (double-hatched bars), as indicated. Sph-1-P (5 μM) was then added and incubations continued for a further 2 h. CTRL indicates incubations in the absence of Sph-1-P (controls). Results are calculated as in Fig. 1, and they are expressed as mean ± S.E.M. of three independent experiments.

whether Ca^{2+} was necessary for stimulation of cortisol secretion by Sph-1-P. Addition of EGTA inhibited this effect significantly, suggesting that extracellular Ca^{2+} is essential in this process (Fig. 3). Although Sph-1-P can also increase cytosolic Ca^{2+} from internal stores by both IP_3-dependent and -independent mechanisms [9,10], it did not stimulate PI-PLC, and the secretion of cortisol was not inhibited further by combined pretreatment of cells with EGTA and the intracellular Ca^{2+} chelators Fura 2-AM, or BAPTA-AM (Fig. 3). The efficiency of Fura 2-AM to chelate Ca^{2+} was tested further by stimulating the cells with the Ca^{2+} ionophore A23187 in the absence of EGTA. A23187 (1 μM) stimulated cortisol secretion by 2.6 ± 0.3 fold (mean ± S.E.M. of three independent experiments) and this was blocked by 10 μM Fura 2-AM. These data suggest that extracellular Ca^{2+}, but not intracellular mobilization of this cation, is relevant for the stimulation of cortisol secretion by Sph-1-P.

Another signaling pathway that can be activated by Sph-1-P is the PLD pathway [8,24]. Fig. 4 shows that Sph-1-P activates PLD in ZF cells. Like stimulation of cortisol secretion, PLD activation was blocked by (a) chelating extracellular Ca^{2+} with EGTA, (b) downregulation of PKC, and (c) treatment with PTX (Fig. 5A–C, respectively). These observations are in agreement with previous work in macrophages [25,26] and Beas-2B cells [27]. Therefore, we hypothesized that PLD might be involved in Sph-1-P-stimulated cortisol secretion. To assess this possibility, cortisol output was examined in the presence of primary alcohols, which reduce the levels of phosphatidate (PA), the product of PLD, by forming phosphatidylalcohols through transphosphatidylation, a reaction catalyzed uniquely by PLD. 'Alcohol trap' experiments have been utilized to establish the role of PLD in various physiological responses. We found that the optimal concentrations of primary alcohols used for PLD determinations, 1% ethanol or 0.3% 1-butanol [5,15,28,29], decreased Sph-1-P-stimulated cortisol secretion by 41 ± 2% and 33 ± 6%, respectively (mean ± S.E.M. of three independent experiments, $P < 0.05$). However, alcohols might have effects on cortisol secretion independently of PLD. Therefore, in some experiments, the cells were stimulated in the presence of 2-butanol, as secondary alcohols are not substrates for PLD [5,30]. Contrary to 1-butanol, concentrations of up to 0.3% of 2-butanol did not affect the stimulation of cortisol secretion by Sph-1-P, suggesting that the inhibitory effect of primary alcohols was caused by decreasing the levels of PLD-derived PA. To further rule out a possible non-specific effect of primary alcohols on cortisol secretion, the cells were stimulated with 22-*R*-hydroxycholesterol, a hydrophilic cholesterol analogue that can bypass the usual signaling pathways necessary for steroid secretion [31]. We found that 22-*R*-hydroxycholesterol (1 μM) stimulated the secretion of cortisol by about 9.6 ± 1.1 fold (mean ± S.E.M., $n = 3$), and this was not significantly altered by 1% ethanol or 0.3% 1-butanol. The implication of PLD in Sph-1-P-induced cortisol secretion was further evaluated by stimulating the cells in the presence of ceramides, which are potent inhibitors of PLD [32]. As expected, C_2-cer-

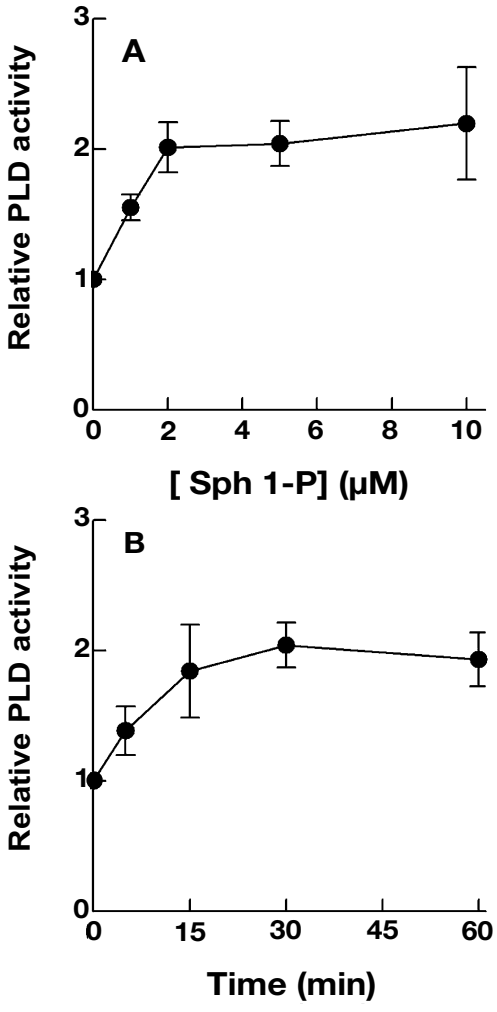

Fig. 4. Sph-1-P stimulates PLD in ZF cells. Cells were treated and labeled as indicated in Section 2. They were stimulated with increasing concentrations of Sph-1-P for 30 min (A), or with 5 μM Sph-1-P for various times (B). Results were calculated as a percentage of the radioactivity present in [^{3}H]phosphatidylethanol compared to that in total lipids, and expressed as the fold stimulation relative to incubations with vehicle. Results are the means ± S.E.M. of three independent experiments.

amide (10 μM) completely blocked the stimulation of PLD by Sph-1-P, whereas the inactive analogue dihydro-C_2-ceramide had no effect (Fig. 6A). Likewise, the stimulation of cortisol secretion by Sph-1-P was completely abolished by C_2-ceramide (100 μM), but not by dihydro-C_2-ceramide (Fig. 6B). The reason why a 100 μM concentration of ceramide was used in these experiments is because cortisol secretion was determined in the presence of a relatively high concentration of BSA (0.2%), which binds ceramide very tightly and makes it unavailable to cells [33]. Taken together, these results suggest that the stimulation of cortisol secretion by Sph-1-P involves PLD activation.

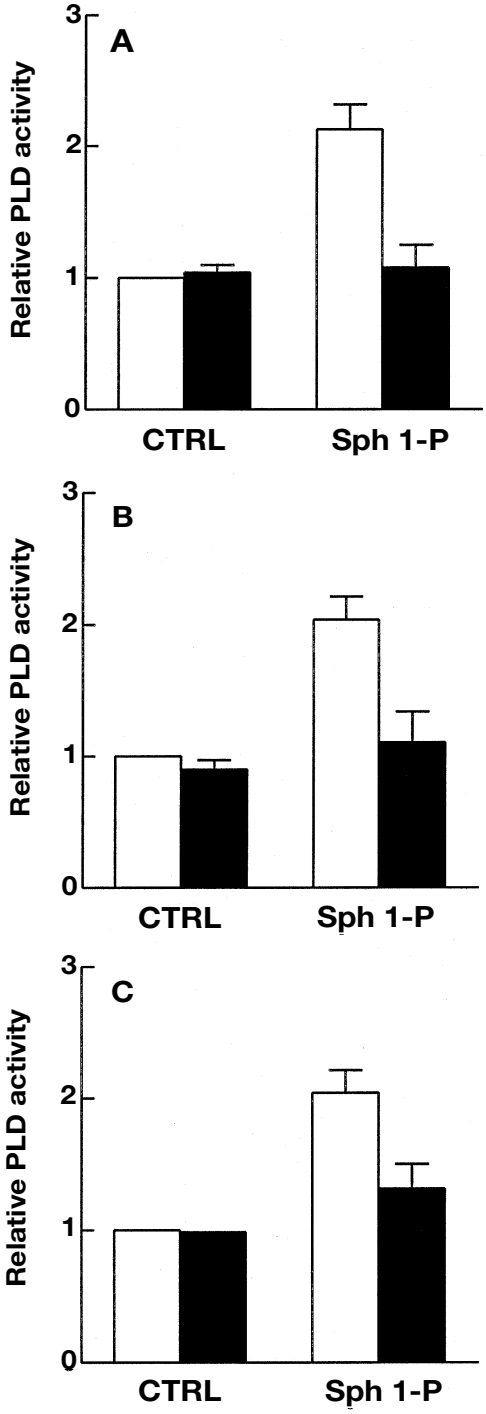

Fig. 5. Effects of EGTA, PMA and PTX on PLD activation by Sph-1-P. Cells were treated and labeled as indicated in Section 2, and they were preincubated with vehicle (empty bars) or with the following additions (solid bars): 5 mM EGTA for 30 min (A), 2 μM PMA for 48 h (B), or 1 μg/ml PTX for16 h (C). Sph-1-P (5 μM) was then added and incubations continued further for 30 min, as indicated. CTRL indicates incubations in the absence of Sph-1-P (controls). In each case, results are calculated as in Fig. 4, and show the means ± S.E.M. of three independent experiments.

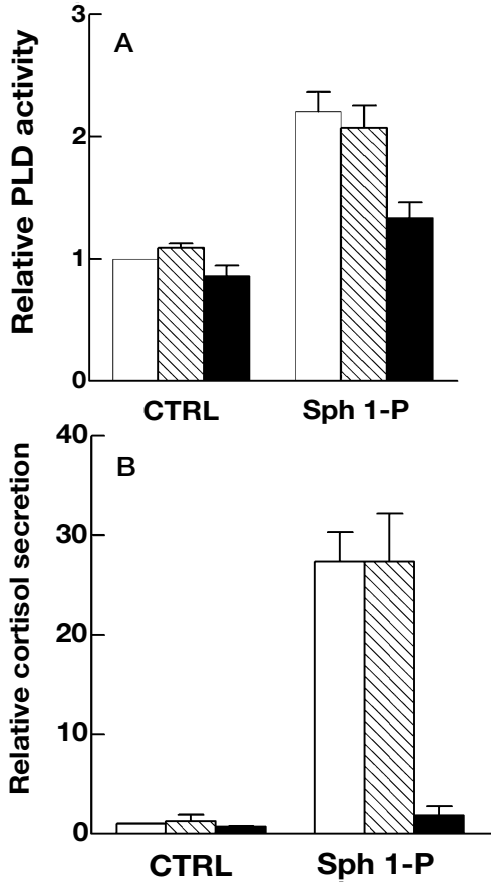

Fig. 6. Inhibition of Sph-1-P-stimulated PLD and cortisol secretion by C_2-ceramide. Cells were treated and labeled as described in Section 2. A: Cells were preincubated with vehicle (empty bars), 10 μM C_2-ceramide (solid bars), or 10 μM dihydro-C_2-ceramide (hatched bars) for 2 h in BSA-free DMEM. Sph-1-P (5 μM) was then added and incubations continued further for 30 min. B: Cells were preincubated with vehicle (empty bars), 100 μM C_2-ceramide (solid bars), or 100 μM dihydro-C_2-ceramide (hatched bars) for 2 h in DMEM supplemented with 0.2% BSA. In each case, Sph-1-P (5 μM) was then added and incubations continued for a further 2 h. CTRL indicates incubations in the absence of Sph-1-P (controls). Values in A are calculated as in Fig. 4, and values in B are calculated as in Fig. 1. Results are expressed as the fold stimulation relative to incubations with vehicle in each case, and they show the means ± S.E.M. of three or four independent experiments.

In conclusion, here we demonstrate that Sph-1-P is a potent stimulator of cortisol secretion in bovine ZF cells. Our results suggest that extracellular Ca^{2+}, Gi/o proteins, PKC, and PLD are all important components in the cascade of events leading to the stimulation of cortisol secretion by Sph-1-P.

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