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Distinct receptor-mediated activities in macrophages for natural ceramide-1-phosphate (C1P) and for phospho-ceramide analogue-1 (PCERA-1)

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Abstract

Ceramide-1-phosphate (C1P) is known as a second messenger regulating a multitude of processes including cell growth, apoptosis and inflammation. Exciting recent findings now suggest that C1P can stimulate macrophages migration in an extra-cellular manner via a G protein-coupled receptor (GPCR). Interestingly, a synthetic C1P analog, named phospho-ceramide analogue-1 (PCERA-1), was recently described as a potent *in-vivo* anti-inflammatory agent, and was suggested to act on macrophages in an extra-cellular manner via a GPCR. Here we summarize and compare the receptor-mediated as well as receptor-independent activities of natural C1P and its synthetic analog. We also provide experimental data in support of distinct C1P and PCERA-1 receptors.

1. Introduction

It is well-established that sphingolipids are crucial metabolites for controlling cell and tissue homeostasis. In particular, ceramides induce cell cycle arrest and are potent inducers of apoptosis. Also, ceramides play crucial roles in the regulation of cell differentiation, and inflammation (Hannun et al., 1986; Hannun, 1994; Hannun and Obeid, 1995; Hannun, 1996; Hannun and Obeid, 2002; Kolesnick, 1994; Kolesnick, 1987; Kolesnick and Hemer, 1990; Kolesnick et al., 2000; Merrill and Jones, 1990; Merrill et al., 1997; Merrill, 2002; Spiegel and Merrill, 1996).

Ceramides are generated either by *de novo* synthesis, or by the action of different sphingomyelinases (SMases), whose activities, enzymology, and compartmentalization have been thoroughly reviewed by others (Cremesti et al., 2002; Goni and Alonso, 2002; Kolesnick et al., 2000). A major metabolite of ceramide is ceramide-1-phosphate (C1P, Fig. 1), which is generated through direct phosphorylation of ceramide by ceramide kinase (CERK) (Bajjalieh et al., 1989; Kolesnick and Hemer, 1990). This enzyme was first shown to be confined to the microsomal fraction, but its location in the cytosol has also been reported (Mitsutake et al., 2004). Recently, Chalfant and co-workers demonstrated that CERK utilizes ceramide transported to the trans-Golgi apparatus by the ceramide transport protein (CERT). Of note, down-regulation of CERT by RNA interference resulted in strong inhibition of newly synthesized C1P, suggesting that CERT plays a critical role in C1P formation (Lamour et al., 2007). However, this observation contrasts with that of Bornancin and co-workers (Boath et al., 2008) who reported that the transport of ceramides to the areas where CERK is located is independent of CERT. The reason for that discrepancy is unknown. Yet, it is possible that different cell types display different expression levels and subcellular distribution of CERK. C1P could also be directly generated from sphingomyelin by the action of SMase D, which cleaves the bond between the choline group and the phosphate rather than between the phospho-choline group and the ceramide. This enzyme is a major component of the venom of a variety of arthropods including spiders of the gender *Loxosceles* (the brown recluse spider), and exists also in the toxins of some bacteria such as *Corynebacterium pseudotuberculosis*, or *Vibrio damsela* (Truett and King, 1993). However, there is no evidence for such activity in mammalian cells.

Contrary to ceramides, C1P (Gomez-Munoz et al., 1995; Gomez-Munoz et al., 1997; Gomez-Munoz, 1998; Gomez-Munoz, 2004) and sphingosine-1-phosphate (S1P) (Spiegel et al., 1993; Spiegel et al., 1996; Spiegel and Merrill, 1996; Spiegel and Milstien, 2002; Spiegel and Milstien, 2003) are potent stimulators of cell proliferation. In addition, C1P regulates apoptosis (Gomez-Munoz, 1998; Gomez-Munoz, 2004), is implicated in the inflammatory response (Chalfant and Spiegel, 2005; Lamour and Chalfant, 2005), and is important in the regulation of phagocytosis (Hinkovska-Galcheva et al., 2005; Hinkovska-Galcheva et al., 1998).

The first part of this review will briefly describe various activities of C1P and will focus on identification of its cellular targets, and in particular – the recently described C1P receptor. The second part of the review will describe the receptor-mediated activities of a synthetic C1P analog, named Phospho-CERamide Analogue-1 (PCERA-1, Fig. 1), and will then compare the two compounds and provide experimental evidence indicating that the two receptors are distinct.

2. C1P regulates inflammation

It was initially reported that ceramides are potent stimulators of arachidonic acid (AA) release. However, recent studies by Chalfant's group suggested that C1P is the actual regulator of AA and prostaglandin (PG) production (Pettus et al., 2003). The importance of CERK, and its product C1P, in cell signaling was highlighted by using specific siRNA to down-regulate its activity in A549 lung adenocarcinoma cells. This treatment inhibited AA release and PGE₂ production in response to a calcium ionophore and to interleukin 1 β (IL-1 β) (Chalfant and Spiegel, 2005; Pettus et al., 2003). Of relevance, C1P was shown to be generated by the actions of either a calcium ionophore or IL-1 β on A549 lung adenocarcinoma cells (Pettus et al., 2003), and M-CSF on bone

marrow-derived macrophages (BMDM) (Gangoiti et al., 2008b). A crucial discovery was the direct implication of C1P in the inflammatory response. Chalfant and co-workers were the first to demonstrate that the mechanism whereby C1P stimulates AA release occurs through direct activation of cPLA₂ (Pettus et al., 2004), and that C1P is a positive allosteric activator of group IV cPLA₂ (Subramanian et al., 2005). C1P was also shown to act in coordination with S1P to ensure maximal production of prostaglandins. It was demonstrated that S1P activates cyclooxygenase-2 (COX-2), which then uses cPLA₂-derived AA as substrate to synthesize prostaglandins (Pettus et al., 2005). For details on the role of C1P in the inflammatory response the reader is referred to excellent recent reviews (Chalfant and Spiegel, 2005; Lamour et al., 2007; Wijesinghe et al., 2007).

3. C1P regulates cell growth and death

Initially, C1P was found to stimulate cell division in rat and mouse fibroblasts (Gomez-Munoz et al., 1995; Gomez-Munoz et al., 1997). This effect was recently also observed in BMDM (Gangoiti et al., 2008b). Specifically, we found that C1P increased DNA synthesis and cell number in macrophages that were pre-incubated with low concentrations of M-CSF to maintain a steady-state cell culture (equal cell growth and death). Like for most growth factors, C1P stimulated cell proliferation through activation of well-established mitogenic pathways including mitogen-activated protein kinase kinase (MEK)/Extracellular regulated kinases 1/2 (ERK1/2), phosphatidylinositol 3-kinase (PI-3K)/protein kinase B (PKB, also known as Akt), and c-Jun terminal kinase (JNK) (Gangoiti et al., 2008b). Additionally, C1P increased expression of a major target of the PI-3K/PKB pathway, glycogen synthase kinase-3 β (GSK-3 β). This action led to up-regulation of cyclin D1 and c-Myc, two important markers of cell proliferation that are well-known targets of GSK-3 β .

In addition to stimulating cell proliferation, C1P is a potent inhibitor of cell death. We found that C1P is present in normal BMDM isolated from healthy mice, whereas C1P levels were substantially decreased in apoptotic macrophages, implying that C1P plays an important role in cell survival (Gomez-Munoz, 2004; Gomez-Munoz et al., 2004). It was found that C1P blocked DNA fragmentation, stimulation of caspases 3 and 9 (Gomez-Munoz et al. 2004), and release of cytochrome c (Gangoiti et al., 2008a), suggesting that the pro-survival effect of C1P was due to inhibition of apoptosis. The latter findings are supported by recent work showing that down-regulation of CERK in mammalian cells reduced growth, promoted apoptosis, and blocked epithelial growth factor (EGF)-induced cell proliferation (Mittra et al., 2007). However, contrary to these observations, Graf and co-workers reported that addition of the cell permeable C₂-ceramide to cells over-expressing CERK led to C₂-C1P formation and apoptosis (Graf et al., 2007). It should however be noted that in contrast to low concentrations of C1P, relatively high concentrations were toxic for fibroblasts or macrophages (Gomez-Munoz, 2006); obviously, over-expression of CERK would potentially increase formation of intracellular C1P, particularly if cells were supplied with a high concentration of exogenous ceramide, resulting in cell toxicity.

Investigation into the mechanism whereby C1P exerts its anti-apoptotic effects demonstrated complete inhibition of acid SMase in intact macrophages incubated with C1P, resulting in reduction of endogenous formation of the pro-apoptotic ceramide, and hence an anti-apoptotic effect (Gomez-Munoz et al., 2004). Importantly, C1P also blocked the activity of acid SMase in cell homogenates, suggesting that inhibition of this enzyme occurs by direct physical interaction with C1P, rather than being mediated through receptor interaction. Acid SMase was also inhibited by S1P in intact macrophages, but unlike C1P the inhibitory effect of S1P did not involve direct interaction with the enzyme (Gomez-Munoz et al., 2003).

Recent work in our lab showed that ceramide levels are increased in apoptotic alveolar NR8383 macrophages (Granado et al., 2009a). However, there was little concomitant stimulation of SMase activities, suggesting a different source for ceramide in these cells. Indeed, it was found

that ceramides were generated by *de novo* synthesis and that one of the pathway's enzymes, serine palmitoyltransferase (SPT), was potently increased under apoptotic conditions in these cells. Of importance, inhibition of SPT activation by treatment with C1P prevented the macrophages from entering apoptosis (Granado et al., 2009a).

It was also demonstrated that PI-3K is a target of C1P in BMDM (Gomez-Munoz et al., 2005). C1P stimulated phosphorylation of protein kinase B (PKB), which is downstream of PI-3K. Both C1P-stimulated PKB phosphorylation and the pro-survival effect of C1P were blocked by the PI-3K inhibitors wortmannin and LY294002. Another relevant finding was that C1P caused I κ B phosphorylation, stimulation of the DNA binding activity of NF- κ B and up-regulation of the expression of anti-apoptotic Bcl-X_L in macrophages (Gangoiti et al., 2008b; Gomez-Munoz et al., 2005). These findings suggest that stimulation of the PI-3K/PKB/ NF- κ B pathway, together with the inhibition of acid SMase, and with SPT inhibition, play crucial roles in the anti-apoptotic effect of C1P in macrophages. Further studies are required to determine whether these pathways are separately modulated by C1P or inter-related. Noteworthy, although we recently identified a specific receptor for C1P (see below), neither the mitogenic nor the anti-apoptotic effect of C1P seem to depend on interaction with the receptor, in macrophages (Gangoiti et al., 2008) or in C2C12 myoblasts (Gangoiti and Gómez-Muñoz, unpublished), as these actions were not inhibited by pertussis toxin (Gangoiti and Gómez-Muñoz, unpublished), in contrast to receptor-mediated cell migration (see below).

It is obvious from the above that the activity of the enzymes involved in ceramide and C1P metabolism must be strictly regulated so as to ensure normal functioning of cells. Any alteration in the balance between ceramides and C1P has consequences on cell life and death and could potentially result in illnesses, including chronic inflammation, neuro-degeneration or tumor development. Investigation into the mechanisms controlling ceramide and C1P levels may be crucial for developing alternative strategies to control metabolic disorders.

4. Evidence for the existence of a specific C1P receptor, implicated in macrophage migration

Macrophages are involved in numerous diseases that are characterized by unregulated chronic inflammation, including autoimmune diseases, atherosclerosis (Rader and Daugherty, 2008) and tumorigenesis (Condeelis and Pollard, 2006). The number of macrophages in tissues is determined by the rates of monocyte recruitment from the bloodstream, the rates of macrophage proliferation and apoptosis, and the rate of macrophage migration and efflux. Recent studies demonstrated that exogenous addition of natural C1P to RAW264.7 macrophages caused cell migration (Granado et al., 2009b). This effect only occurred when C1P was added exogenously to the cells in culture, and was independent of the intra-cellular formation of C1P. It was thus concluded that C1P stimulates cell migration in an extra-cellular manner via a specific receptor. The receptor has a relatively high K_d for C1P (7.8 μ M), and therefore relatively high concentrations of C1P were required for optimal activation of the receptor. However, it should be kept in mind that in those studies C1P was added to the cells in a vesicular form (sonicated in water), and therefore the actual C1P concentration that is available for receptor binding is expected to be much lower than the total concentration. Additionally, the tight binding of C1P to albumin and other serum proteins further reduces its free concentration which is available to the cells.

Importantly, we found that pertussis toxin blocked C1P-induced macrophages migration and that C1P increased GTP γ S binding to macrophages membranes. These findings indicate that the C1P receptor belongs to the GPCR super-family and that it is specifically coupled to G_i proteins. Activation of the receptor upon ligation with C1P led to phosphorylation of ERK1/2 and PKB, and specific inhibitors of either of these pathways completely abolished C1P-stimulated macrophage migration. In addition, C1P stimulated the DNA binding activity of NF- κ B via both ERK1/2 and PKB, and specific blockade of this transcription factor also resulted in complete inhibition of macrophage migration. Finally, like cell migration, activation of these signaling pathways was

blocked by pertussis toxin, further demonstrating that cell migration is induced by C1P via a G_i-coupled receptor, which is upstream to the MEK/ERK1/2, PI-3K/PKB and NFκB pathways (Granado et al., 2009b). Although these pathways are also involved in the mitogenic and/or anti-apoptotic effects of C1P, their activation are independent of interaction with the C1P receptor, possibly because they belong to a pool of kinases that is spatially distinct to that involved in C1P-stimulated-cell migration (Gómez-Muñoz et al., 2009).

The field of phospholipid-binding receptors has been attracting increasing attention in recent years since the discovery of multiple G-protein-coupled receptors for the endogenous phospholipid mediators, S1P and lysophosphatidic acid (LPA) (Alvarez et al., 2007; Anliker and Chun, 2004; Rosen and Goetzl, 2005). Using radiolabelled C1P we have verified that S1P and LPA do not compete with C1P on binding to its receptor (Granado et al., 2009b). Thus, it can be concluded that the C1P receptor represents a novel phospholipid-binding receptor.

The existence of a cell-surface receptor for C1P in macrophages implies that C1P is secreted and then acts in an autocrine manner on the same cell or in a paracrine manner on neighboring cells. Indeed, secretion of C1P along the secretory pathway and into the medium has been monitored in BMDM (Boath et al., 2008). The mechanism of C1P secretion remains to be explored.

5. Evidence for a PCERA-1 receptor

The pharmaceutical company ONO has described the synthesis, and application in a sepsis model, of a phospholipid-like molecule, 1-methyl-2-(3-methoxyphenyl)-2-(octanoylamino)ethyl-disodium-phosphate. Following the discovery of a lead compound by *in-vivo* screening in rodents, this drug was rationally developed as a potent *in-vivo* suppressor of LPS-induced TNF-α secretion (Matsui et al., 2002b; Matsui et al., 2002d; Matsui et al., 2002e; Matsui et al., 2003). A thorough structure-activity relationships study has demonstrated that both the phosphate (Matsui et al., 2002c; Matsui et al., 2002d) and the lipidic (Matsui et al., 2002c; Matsui et al., 2002d) portions of PCERA-1 are required for activity. Interestingly, we noticed that these moieties and the correct spacing in-between them, are present also in the natural C1P (Fig. 1). Thus, we have named this molecule Phospho-CERamide Analogue-1 (PCERA-1, Fig. 1). Importantly, the correct stereochemistry is essential for the activity of PCERA-1, as the [1S,2R] stereoisomer is two orders of magnitude more potent than the [1S,2S] stereoisomer (Matsui et al., 2002d), indicating that this phospholipid-like molecule binds a protein target, rather than affects membrane structure in a non-specific manner. The identical dose-response curves obtained for the independent modulation of TNF-α and IL-10 production in LPS-stimulated macrophages by PCERA-1, suggests that a single protein target mediates both activities (Goldsmith et al., 2008).

The identity of the protein target of PCERA-1 is yet to be determined. However, multiple experimental evidences implicate a cell surface receptor expressed in macrophages, and specifically point the finger at a GPCR. These evidences are:

- 1) The negative charge of a phosphate group presents an obstacle for membrane permeability. However, Boudker and Futerman demonstrated that exogenous C1P can be de-phosphorylated by an extra-cellular phosphatase, and the resulting ceramide enters the cell and becomes enriched in the Golgi apparatus (Boudker and Futerman, 1993). Once inside the cell, ceramide can either be active as is, be re-phosphorylated by an intra-cellular ceramide kinase (Lamour and Chalfant, 2008; Saxena et al., 2008) or be converted to a different ceramide metabolite (Worgall, 2007). Yet, the α-methyl group of PCERA-1 (Fig. 1) protects the phosphate from being enzymatically removed as shown by *in-vitro* stability assays with tissue homogenates (Matsui et al., 2002c; Matsui et al., 2002d). Moreover, since de-phosphorylation of PCERA-1 would give rise to a cell-permeable CERA-1, one can predict that if PCERA-1 crosses the cell membrane by the mechanism suggested above for C1P, then exogenous CERA-1 should be at least as active as

exogenous PCERA-1. However, CERA-1 was found to be essentially inactive, both *in-vivo* (Avni et al., 2009; Matsui et al., 2002c; Matsui et al., 2002d), and *in-vitro* (Goldsmith et al., 2008), suggesting that PCERA-1 acts in an extra-cellular manner. It should be noted however that exogenous phospholipids may also enter the cell by alternative mechanisms such as endocytosis (Boudker and Futerman, 1993) or via the scavenger receptor (Adachi and Tsujimoto, 2006). While these mechanisms may facilitate cell permeability for PCERA-1, their physiological relevance to cytokine modulation is refuted by the following observations.

2) Permeability and subsequent intra-cellular activity of PCERA-1 would predict resistance to washing. However, it was found that only macrophages co-incubated with LPS and PCERA-1 show modulated TNF- α production (relative to LPS-stimulated macrophages), and that pre-incubation is not advantageous for PCERA-1 activity (Goldsmith et al., 2008). In that sense PCERA-1 resembled PGE₂, which suppresses TNF- α production in an extra-cellular manner via membrane receptors, and differed from the cell-permeable dexamethasone, which suppresses TNF- α production via a nuclear receptor, and was therefore resistant to washing (Goldsmith et al., 2008).

3) PCERA-1 rapidly elevates intra-cellular cAMP level in macrophages (Goldsmith et al., 2008), suggesting it activates a GPCR upstream to adenylyl cyclase (AC).

4) Activation of AC by PCERA-1 was also demonstrated in macrophages membranes, confirming that the protein target of PCERA-1 resides in the cell membrane rather than being a cytoplasmic modulator of cAMP level (Philosoph and Zor, unpublished).

5) The activation of AC in membranes by PCERA-1 was dependent on the presence of GTP, in contrast to forskolin-induced cAMP formation (Philosoph and Zor, unpublished). This finding is indicative of a G-protein-mediated activation of AC.

Taken together, the above findings argue in favor of a GPCR as the PCERA-1 receptor. The anti-inflammatory activity of PCERA-1 has been demonstrated in the mouse RAW264.7 macrophage cell line (Goldsmith et al., 2008) as well as in primary mouse macrophages (Avni et al., 2009). Of note, isolated blood monocytes were inert to PCERA-1 but acquired sensitivity to PCERA-1 upon M-CSF-induced differentiation into macrophages (Avni et al., 2009). It is thus suggested that expression of the PCERA-1 receptor is up-regulated as monocytes differentiate into macrophages.

We have shown that the endogenous phospholipid mediators, S1P and LPA, do not function as agonists or as antagonists for the activities of PCERA-1 in macrophages (Goldsmith et al., 2008). Of relevance, LDL-derived oxidized phospholipids have been shown to induce formation of the second messenger cAMP (Cole et al., 2003). More recently, the PGE₂ receptor EP2 has been shown to specifically bind a LDL-derived oxidized phospholipid (Li et al., 2006). We have demonstrated that an EP2 antagonist was unable to antagonize the activity of PCERA-1 in macrophages (Avni et al., 2009). Thus, it can be concluded that the anti-inflammatory PCERA-1 modulates production of key cytokines in activated macrophages via a novel GPCR, distinct of the currently known phospholipid-binding receptors for S1P, LPA and oxidized phospholipids. As will be discussed below, the PCERA-1 receptor is also distinct of the newly-suggested C1P receptor.

6. The receptor-mediated activities of PCERA-1

PCERA-1 was initially described as an *in-vivo* suppressor of TNF α production in LPS-challenged mice (Matsui et al., 2002b; Matsui et al., 2002d; Matsui et al., 2002e; Matsui et al., 2003). We have extended these studies to identify the target cell, the effect on production of other inflammatory mediators, and the mechanism of action of PCERA-1. The mechanistic study was enabled by the finding that macrophages, both cultured and primary, are a major cell target of PCERA-1 (Avni et al., 2009; Goldsmith et al., 2008). The ability of a PCERA-1 derivative to

reduce mortality and increase survival in a mouse LPS-induced sepsis model was attributed solely to the reduced production of the pro-inflammatory cytokine TNF α (Matsui et al., 2002c). However, we found that in addition, PCERA-1 inhibited production of the p40 subunit of the pro-inflammatory cytokines IL-12 and IL-23, and elevated production of the anti-inflammatory cytokine IL-10, in LPS-challenged mice and in LPS-stimulated macrophages (Avni et al., 2009; Goldsmith et al., 2008). These activities were found to be independent of each other (Avni et al., 2009; Goldsmith et al., 2008). PCERA-1 suppressed TNF α production, not only in LPS-stimulated mice and macrophages, but also when other toll-like receptors (TLRs) initiated the inflammatory stimulus (Goldsmith et al., 2008). Interestingly, induction of IL-10 in macrophages was minimal in the presence of PCERA-1 alone, modest in the presence of the inflammatory stimulus alone, but synergistic in the presence of both PCERA-1 and a TLR agonist (Goldsmith et al., 2009). Thus, cytokine modulation by PCERA-1 is restricted to activated macrophages and does not occur in resting macrophages.

Activation of the cAMP pathway appears to be the major route by which PCERA-1 modulates cytokine production. Incubation of RAW264.7 macrophages with PCERA-1 leads to a rapid increase in intra-cellular cAMP level (Goldsmith et al., 2008) and subsequent phosphorylation of CREB (Avni and Zor, unpublished). Moreover, a PKA inhibitor blocked the effect of PCERA-1 on IL-10 (Goldsmith et al., 2009) and TNF α (Avni and Zor, unpublished) production. PCERA-1 also increases phosphorylation, and hence activation, of p38 MAP kinase, both in resting macrophages and in LPS-stimulated macrophages (Goldsmith et al., 2009). Interestingly, p38 inhibitors blocked IL-10 production by LPS and PCERA-1 (Goldsmith et al., 2009), and yet intensified the suppressive effect of PCERA-1 on LPS-induced TNF α production (Levy-Rimler and Zor, unpublished). Thus, p38 activity is required for the positive effect of PCERA-1 on IL-10 production, whereas it interferes with the negative effect of PCERA-1 on TNF α production. Modulation of TNF α and IL-10 production by PCERA-1 occurs at both the protein and mRNA levels (Avni et al., 2009; Goldsmith et al., 2008), suggesting that PCERA-1 acts at the level of transcription or mRNA stability. Synergistic IL-10 production by PCERA-1 and LPS was demonstrated on both the endogenous IL-10 gene and on a transiently transfected IL-10 promoter-driven reporter gene (Goldsmith et al., 2009). This finding indicates that PCERA-1 acts at the transcriptional level of IL-10 expression.

The anti-inflammatory direction that PCERA-1 confers on cytokine production in LPS-stimulated macrophages has raised the question whether PCERA-1 affects also the production of the pro-inflammatory mediator PGE₂. We found that PCERA-1 inhibited LPS-induced cPLA₂ activation and subsequent PGE₂ production (Goldsmith and Zor, unpublished). Taken together, the reciprocal effects of PCERA-1 on production of pro-inflammatory (TNF α , IL-12, IL-23, PGE₂) and anti-inflammatory (IL-10) mediators, sum up to a robust anti-inflammatory activity.

7. Functional comparison of C1P and PCERA-1

C1P and PCERA-1 display distinct sets of receptor-mediated cellular activities in macrophages, as described below and schematically illustrated in Fig. 2.

- 1) C1P elicited cell migration (Granado et al., 2009b), whereas PCERA-1 modulated LPS-induced TNF α and IL-10 production (Goldsmith et al., 2008).
- 2) C1P elevated ERK1/2 and JNK (but not p38) phosphorylation (Granado et al., 2009b), whereas PCERA-1 elevated p38 (but not ERK1/2 and JNK) phosphorylation (Goldsmith et al., 2009).
- 3) C1P activated the NF κ B and PI-3K pathways (Granado et al., 2009b), whereas PCERA-1 neither significantly affected nuclear translocation of NF κ B (Goldsmith et al., 2008), nor activated PI-3K (Avni and Zor, unpublished).

- 4) C1P reduced basal AC activity (Goldsmith et al., 2008), presumably via the G_i protein (Granado et al., 2009b), whereas PCERA-1 activated AC (Goldsmith et al., 2008), presumably via the G_s protein.

The receptor-mediated activities of C1P and PCERA-1 have both been demonstrated in RAW264.7 macrophages (Goldsmith et al., 2008; Granado et al., 2009b). Taken together with the structural similarity between the two compounds (Fig. 1), the possibility of a common receptor should be discussed. The end cellular effects reported for C1P (cell migration via the NF κ B pathway) and for PCERA-1 (cytokine modulation via the cAMP pathway) are distinct, and thus are likely to originate from distinct receptors. Yet, these cellular effects may possibly also represent various activities of a single receptor that were found for one compound but overlooked for the other compound. Alternatively, the distinct cellular activities may possibly be induced by receptor conformations unique for each ligand, a phenomenon named agonist-directed trafficking of response (Prather, 2004). The β -adrenergic receptor (β -AR) represents a relevant example. The specific β -AR inverse agonist propranolol not only blocks G_s -mediated AC activation, but also activates the ERK pathway via β -arrestin (Azzi et al., 2003). Thus, propranolol is a dual efficacy ligand – acts as an inverse agonist for one β -AR activity, and as an agonist for another β -AR activity (Azzi et al., 2003). Finally, as a third alternative, it is possible that while C1P acts as an agonist of a G_i -coupled receptor (Granado et al., 2009b), PCERA-1 simply acts as an inverse agonist of that receptor. In that scenario, PCERA-1 blocks constitutive G_i -mediated AC inhibition, leading to an observed elevated cAMP level resulting from the constitutive activity of G_s -coupled receptors.

In light of the above, antagonism between C1P and PCERA-1 needs to be examined in order to distinguish between the possibilities of a common receptor versus distinct receptors. Figure 3 shows that PCERA-1 suppressed production of TNF α and elevated production of IL-10 in LPS-stimulated RAW264.7 macrophages. We found that a synthetic C_8 -C1P was neither able to significantly mimic, nor able to block, these activities (Fig. 3). Of note, the same preparation of C_8 -C1P reduced basal cAMP formation in the macrophages, attesting to C1P receptor activity (Goldsmith et al., 2008). These results indicate that C1P can not bind the PCERA-1 receptor, but do not preclude binding of PCERA-1 to both receptors. However, preliminary results further show that PCERA-1 does not affect NF κ B activation by natural C1P (Avni and Zor, unpublished). Taken together, these results suggest that C1P and PCERA-1 act via distinct receptors, rather than a shared receptor.

Interestingly, C1P and PCERA-1 display opposite effects on PLA₂ activation and subsequent PGE₂ production. C1P was shown to directly activate PLA₂, implying that it has both receptor-dependent and receptor-independent activities (Pettus et al., 2004; Wijesinghe et al., 2008). PCERA-1 on the other hand, blocks LPS-induced PLA₂ activation and inhibits subsequent PGE₂ production (Goldsmith and Zor, unpublished). It remains to be explored whether this activity of PCERA-1 is receptor-dependent and cAMP-mediated, or whether PCERA-1 can also directly bind to PLA₂ in a receptor-independent manner.

8. Structural comparison of C1P and PCERA-1

What is the molecular feature that distinguishes between C1P and PCERA-1?

The length of the amide-linked fatty acid chain may be a factor in PCERA-1 receptor recognition since addition of 3 carbons (or reduction of 2 carbons) to the 8-carbon long chain of a PCERA-1 derivative reduced its *in-vivo* potency by an order of magnitude (Matsui et al., 2002a; Matsui et al., 2002b). However, a synthetic short chain C_8 -C1P, which has an identical chain length to PCERA-1, can bind the C1P receptor on the one hand (Granado et al., 2009b), but can not antagonize PCERA-1 on the other hand (Fig. 3). Thus, since both natural (C_{16} - C_{18}) and synthetic (C_8) C1P were demonstrated to be selective for the C1P receptor (and not for the

PCERA-1 receptor), chain length is unlikely to be the important factor in recognition of either receptor.

As mentioned above, the *in-vivo* potency of the [1*S*,2*R*]-PCERA-1 stereoisomer is two orders of magnitude higher than that of the [1*S*,2*S*] stereoisomer (Matsui et al., 2002d). Thus, the configuration at the carbon 2 position (Fig. 1, numbering from the phosphate moiety) is clearly critical for activity. In contrast, the configuration (following sequence priority rules) of natural C1P is [2*S*] (Wijesinghe et al., 2005). However, the phosphate and the amide groups are similarly oriented in space relative to the sphingoid chain of C1P, and to the aromatic ring of PCERA-1. Thus, receptor selectivity can not be explained on the basis of stereochemical reasoning.

Obviously, the sphingoid chain of C1P differs from the aromatic ring of PCERA-1 (Fig. 1). A structure-function study of PCERA-1 derivatives has shown relative tolerability to hydrophobic modification, but low tolerability to polar modification of the aromatic ring (Matsui et al., 2002a). Accordingly, the secondary hydroxyl group that is present only in C1P may participate in hydrogen bonding in the C1P receptor binding site, and at the same time repel a hydrophobic residue that may be present in the PCERA-1 receptor binding site. Thus, it is conceivable that the aromatic ring of PCERA-1 and the sphingoid chain (and in particular the hydroxyl group) of C1P confer receptor selectivity.

9. Open questions and concluding remarks

The field of phospholipid-binding receptors and the field of ceramide signaling have unexpectedly been opened up with recent evidences coming from our laboratories for the existence of novel receptors belonging to the GPCR family for C1P and for the C1P analog PCERA-1. We have compared here the signaling properties of the two compounds, and supplied experimental data in support of distinct receptors (Fig. 2). The most intriguing task that the field is now confronted with is the identification of the C1P and PCERA-1 receptors. Major questions are: what is the endogenous ligand of the PCERA-1 receptor? What is the tissue distribution of these receptors and what activities do the compounds have outside the immune system? Does the C1P receptor mediate some of the effects of C1P on proliferation and apoptosis? Which specific natural C1P variants bind the C1P receptor? What are the stimuli and what is the mechanism for C1P secretion? Do C1P and PCERA-1 overlap in non-receptor C1P targets such as SMase or PLA₂? These are only several of the exciting questions waiting to be addressed.

C1P may have diverse effects on inflammation by stimulating macrophages migration (and hence inflammation resolution) on the one hand and by production of lipid mediators on the other hand. In contrast, C1P displays an anti-inflammatory character by suppressing production of pro-inflammatory cytokines and lipid mediators, and enhancing production of the anti-inflammatory cytokine IL-10. It will not be a surprise if additional effects on inflammation will be found for these compounds. In light of the distinct receptors and receptor activities for C1P and PCERA-1, it is expected that research into the open questions exemplified above will lead to important insights in the field of cellular signaling and will pave the way for the development of novel therapeutics to inflammation-linked diseases.

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Abbreviations

PCERA-1, phospho-ceramide analogue-1; CERA-1, ceramide analog-1; TLR, toll like receptor; LPS, lipopolysaccharide; TNF- α , tumor necrosis factor alpha; IL-10, interleukin-10; S1P, sphingosine-1-phosphate; LPA, lysophosphatidic acid; C1P, ceramide-1-phosphate; GPCR, G-protein coupled receptor; AC, adenylyl cyclase; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; ERK, extracellular signal-regulated kinase; JNK, c-jun N-terminal kinase; SMA, sphingomyelinase; SPT, serine palmitoyltransferase; PKA, protein kinase A; PKB, protein kinase B; PLA₂, phospholipase A₂; AA, arachidonic acid; PGE₂, prostaglandin E₂; COX, cyclooxygenase; CERK, ceramide kinase; PI-3K, phosphatidylinositol 3-kinase; EGF, epithelial growth factor; BMDM, bone marrow-derived macrophages.

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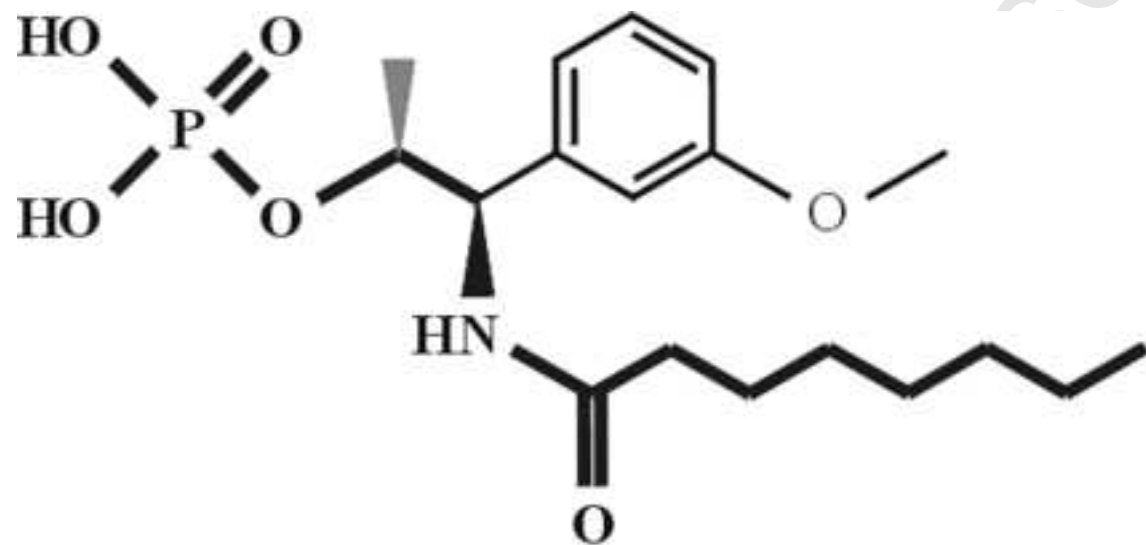
Figure legends

Fig. 1. Structures of PCERA-1 and C1P. The identical parts are highlighted by bold lines and letters.

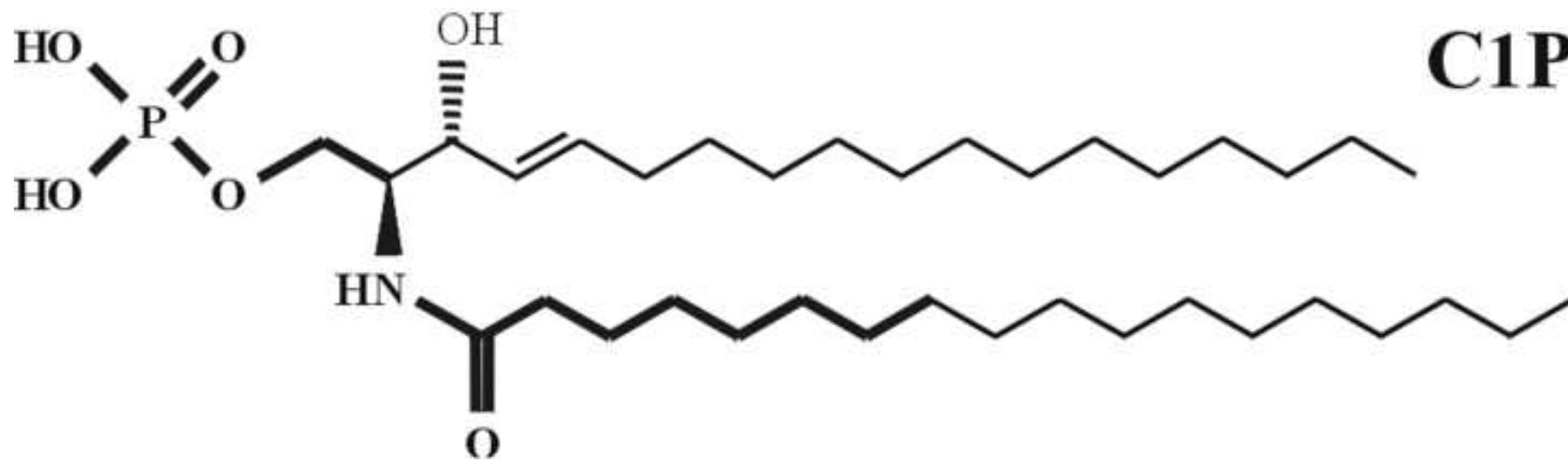
Fig. 2. A proposed model for the receptor-mediated signaling pathways of C1P and PCERA-1 in macrophages.

Fig. 3. C1P does not affect TNF- α and IL-10 production modulation by PCERA-1. Mouse macrophage RAW264.7 cells were incubated at 37°C for 2 hours with LPS (100 ng/ml) and with PCERA-1 (0.1 μ M) and/or C₈-C1P (10 μ M). TNF- α (A) and IL-10 (B) release to the medium were measured by ELISA. Each data point represents the mean \pm S.D. (n=6). The vehicle, which included 0.5% ethanol, had no significant effect on cytokine release. The results are representative of 3 independent experiments. Synthetic C₈-C1P (Avanti Polar Lipids, Alabaster, AL) was initially dissolved in ethanol and then diluted in culture media containing 4% fatty acid-free BSA. The activity of C₈-C1P was verified by its ability to reduce basal cAMP level. Similar results were obtained with 100 μ M C₈-C1P, or with sonicated natural C1P rather than C₈-C1P. Cell culture conditions, reagents and PCERA-1 synthesis were previously described (Goldsmith et al., 2008).

Figure 1



PCERA-1



C1P

Figure 2

