Ceramide-activated protein phosphatase-2A activity in insulin-secreting cells

Anjaneyulu Kowluru[a,b,*], Stewart A. Metz[a,b]

[a]*Research and Medical Services, William S. Middleton Memorial VA Medical Center, 2500 Overlook Terrace, Madison, WI 57305, USA*
[b]*Department of Medicine and Section of Endocrinology, University of Wisconsin School of Medicine, Madison, WI 53717, USA*

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Abstract Okadaic acid (OKA)-sensitive phosphatase (PP2A) activity may modulate nutrient-induced insulin secretion from pancreatic β cells [Kowluru et al., Endocrinology 137 (1996) 2315–2323]. Ceramides, a new class of lipid second messengers may regulate PP2A [Dobrowsky and Hannun, J. Biol. Chem. (1992) 267, 5048–5051], and might play a role in cytokine-mediated apoptosis in β cells [Sjöholm, FEBS Lett. 367 (1995) 283–286]. Therefore, we investigated the regulation of PP2A-like activity by ceramides in isolated β (HIT-T15 or INS-1) cells. Cell-permeable (C2, C6 or C18) ceramides stimulated OKA-sensitive (but not -insensitive) phosphatase activity in a concentration-dependent manner (0–12.5 μM), with maximal stimulation (+50–100%) at < 12.5 μM. C2-dihydroceramide (a biologically inactive analog of C2 ceramide) failed to augment PP2A-like activity. Stimulatory effects of ceramides do not appear to be mediated via activation of the carboxyl methylation of the catalytic subunit of protein phosphatase 2A, since no effects of ceramides (up to 25 μM) were demonstrable on this parameter. These data identify a ceramide-activated protein phosphatase as a possible locus at which ceramides might exert their effects on β cells leading to altered insulin secretion, and decreased cell viability followed by apoptotic cell demise.

Key words: Pancreatic β cell; Protein phosphatase 2A; Okadaic acid; Ceramide; Carboxyl methylation; Apoptosis

1. Introduction

Glucose-induced insulin secretion from the pancreatic β cell involves the generation of soluble second messengers, including ions, cyclic nucleotides, lipid hydrolytic products of phospholipases, adenine and guanine nucleotides [1,2]. More recently, ceramides, a new class of lipid second messengers, have been implicated in cell signalling in different cell types [3–7]. Ceramides are formed from the hydrolysis of sphingomyelin by membrane-bound sphingomyelinase [3–7]. Recently, localization of such an enzyme activity was reported in isolated rat islets, mouse islets and clonal β cells [8]. Emerging data indicate that receptor activation by cytokines (e.g. tumor necrosis factor [TNF] or interleukin-1β [IL-1β]) results in the activation of a membrane-associated sphingomyelinase resulting in the hydrolysis of sphingomyelin to yield ceramides and phosphocholine [3–7].

There are at least two reports of regulation by ceramides of β cell function.

Sjöholm [9] reported that exposure of pancreatic β cells to synthetic ceramides or purified sphingomyelinase resulted in marked reduction in insulin production and mitogenesis. These effects were mimicked by IL-1β, indicating that ceramides might play a key regulatory role in cytokine-mediated signalling in the β cell. More recently, Major and coworkers [10] provided evidence to indicate that long-term exposure (24–96 h) of isolated β cells to C2- or C6-ceramides (but not C2-dihydroceramide, a biologically inactive ceramide) significantly reduced glucose- and carbachol-induced insulin secretion from these cells. Furthermore, the metabolic viability of these cells was also markedly attenuated following exposure to ceramides [10].

Recent evidence obtained using glioma cells and rat brain cells [11,12] also indicates that ceramide-mediated signalling involves activation of a phosphoprotein phosphatase (commonly referred to as the ceramide-activated protein phosphatase; CAPP). Based upon its cytosolic distribution, sensitivity to low nM concentrations of OKA, and insensitivity to divalent cations, CAPP was assigned to the family of okadaic acid (OKA)-sensitive phosphatase (PP2A) [3]. Several recent studies, including our own [13], have provided evidence to suggest that PP2A may play a regulatory role in β cell function. We also reported [13] that the catalytic subunit of PP2A (PP2Ac) undergoes methylation-demethylation at its C-terminal leucine (Leu-309), and that ebelactone, an inhibitor of PP2Ac demethylation, markedly reduced nutrient-induced insulin secretion from normal rat islets. Taken together, these data seem to suggest a key modulatory role for PP2A in insulin secretion. The present study was, therefore, undertaken to examine whether the β cell PP2A is an effector protein in the ceramide signalling pathway and, if so, whether the effects of ceramide on PP2A are mediated via the carboxyl methylation of PP2Ac.

2. Materials and methods

S-Adenosyl-L-[^{3}H]methionine (73 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were obtained from NEN-DuPont (Boston, MA). Histone (type II-AS; calf thymus), cAMP, and purified cAMP-dependent protein kinase (rabbit muscle) were purchased from Sigma (St. Louis, MO). OKA was obtained from LC Laboratories (Woburn, MA) or Sigma or Alexis (San Diego, CA). Ceramides and dihydroceramides were purchased from Sigma or Calbiochem (San Diego, CA). Stock solutions of ceramides were prepared in ethanol and diluted accordingly in the reaction buffer as indicated in the text. Appropriate diluents were included in the control tubes. All other reagents used were of highest purity available.

2.1. Sources of β cells and isolation of subcellular fractions

Pancreatic islets were isolated from male Sprague-Dawley rats (300–400 g body weight) by collagenase digestion method as previously described [13]. HIT cells (passage number 72) were kindly provided by Dr. Paul Robertson, University of Minnesota Medical School (Minneapolis, MN). INS-1 cells were generously provided by Dr.

*Corresponding author. H4/568, Clinical Sciences Center, 600 Highland Avenue, Madison, WI 53792, USA. Fax: (1) (608) 263-9983. E-mail: akowluru@facstaff.wisc.edu

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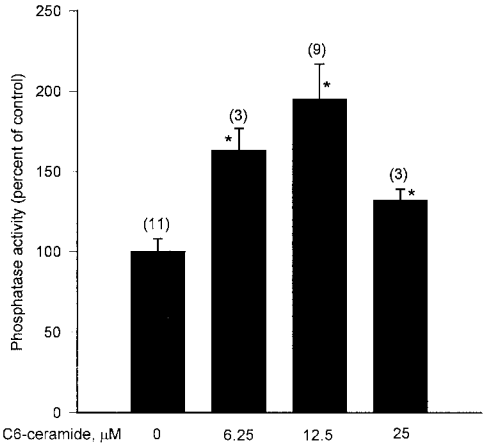

Fig. 1. Stimulation of PP2A activity by hexanoyl ceramide: concentration dependence. PP2A activity was measured in INS cell cytosolic fraction in the presence of increasing concentrations (0–25 μM) of C6-ceramide. Data are expressed as percent of control activity (representing 2–3 pmol of ^{32}P released/10 min) observed in the absence of ceramide and are means ± S.E.M. of the number of determinations indicated in parentheses. $*P < 0.05$ vs. control.

Claes Wollheim, University of Geneva (Geneva, Switzerland). Both HIT and INS-1 cells were cultured as described previously [13]. The cytosolic fraction from insulin-secreting cells was obtained by centrifugation of homogenate at 105 000 × *g* for 90 min (Ultima TL-100, Beckman, Palo Alto, CA) as previously described [13].

2.2. Carboxyl methylation of PP2Ac in cell-free preparations

The reaction mixture (100 μl) consisted of 100 mM Tris-HCl (pH 7.5), 1 mM EDTA, 4 mM DTT, and β cell protein (10–20 μg). The reaction was initiated by the addition of [^{3}H]*S*-adenosyl methionine and continued for 30 min at 37°C. Modulators were present in the reaction mixture where indicated in the text. The reaction was terminated by the addition of SDS-PAGE sample buffer, and labelled proteins were separated by SDS-PAGE and degree of methylation was quantitated by vapor-phase equilibration assay as we described recently [13].

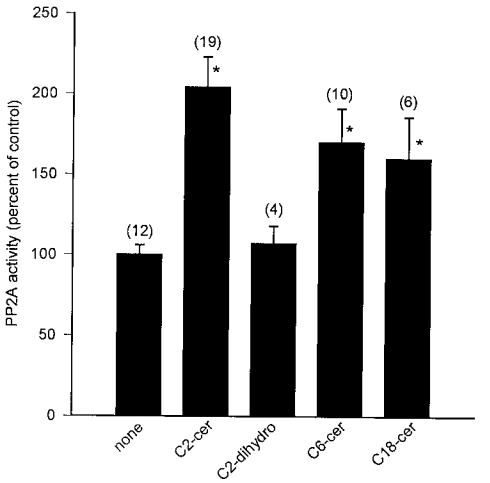

Fig. 2. Specificity of ceramide stimulation of PP2A activity in INS cell cytosol. OKA-sensitive phosphatase activity was measured in INS cell cytosolic fraction using [^{32}P]histone as the substrate in the presence of various ceramides (12.5 μM) as indicated. Data are expressed as percent of control activity (representing 2–3 pmol of ^{32}P released/10 min) observed in the absence of ceramides and are means ± S.E.M. of the number of individual determinations as indicated in parentheses. $*P < 0.05$ vs. control.

2.3. Quantitation of protein phosphatase activity

This was carried out by quantitating the rates of hydrolysis of ^{32}P-labelled histone according to the method of Sjöholm et al. [14] with minor modifications, as described in [13]. Briefly, the reaction mixture (in a total volume of 100 μl) consisted of 100 mM Tris-HCl (pH 7.4), 2 mM DTT, 1 mM EDTA and β cell protein (typically 10–20 μg). The reaction was started by the addition of ^{32}P-labelled histones (corresponding to 1×10^5 cpm) and carried out for 10 min at 30°C. It was terminated by adding 100 μl of 20% trichloroacetic acid. To this, 50 μl BSA (10 mg/ml) was added as a carrier protein, and the mixture was centrifuged at 12 000 × *g* for 5 min. 150 μl of the supernatant was aliquoted into scintillation vials containing 5 ml scintillant, and the radioactivity was determined by scintillation spectrometry. For control studies, β cell proteins were omitted from the reaction mixture, and the values representing non-specific hydrolysis were subtracted from the original values for the calculation of PP2A activity. PPase 1-like activity was calculated as the activity remaining in the presence of okadaic acid and the PP2A-like activity was calculated as the difference between total activity (blanks subtracted) and the PPase 1-like activity [14].

2.4. Other methods

The protein concentration in samples was assayed by a dye-binding method described previously [15], using BSA as a standard. SDS-PAGE (12% acrylamide gels) was carried out as previously described [13].

3. Results

C6-ceramide stimulated the OKA-sensitive PP2A activity in INS cell cytosolic fraction in a concentration-dependent manner (Fig. 1). Maximal stimulation (+100%) was demonstrable at 12.5 μM. Higher concentrations (25 μM) were less stimulatory (Fig. 1). Other ceramides, such as acetyl (C2)- and stearoyl (C18)-ceramides also stimulated PP2A-like activity in INS cell cytosol (Fig. 2) in the following rank order: C2 > C6 = C18 > C2-dihydroceramide = control. Similar stimulatory effects were also demonstrable in HIT cell and normal rat islet cytosolic fractions (data not shown). C2-Dihydroceramide, an inactive analog of C2-ceramide [11,12], failed to stimulate PP2A activity (Fig. 2). These data indicate specific regulation of PP2A activity by biologically active ceramides.

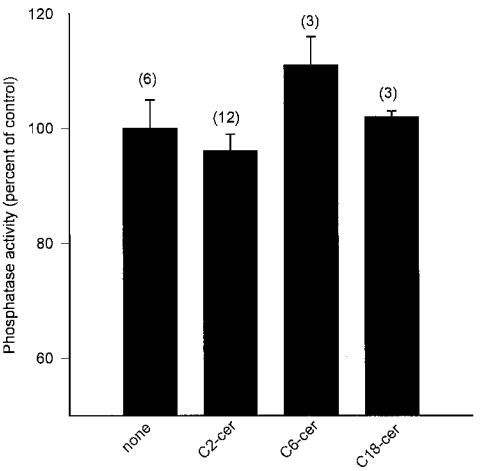

Fig. 3. Lack of effect of ceramides on OKA-insensitive phosphatase activity. OKA-insensitive phosphatase activity was measured in INS cell cytosolic fraction using [^{32}P]histone as the substrate (see Section 2) in the presence of various ceramides (12.5 μM) as indicated. Data are expressed as the percent of control activity (representing 0.7–1.0 pmol ^{32}P released/10 min) observed in the absence of ceramides and are means ± S.E.M. of the number of individual determinations as indicated in parentheses.

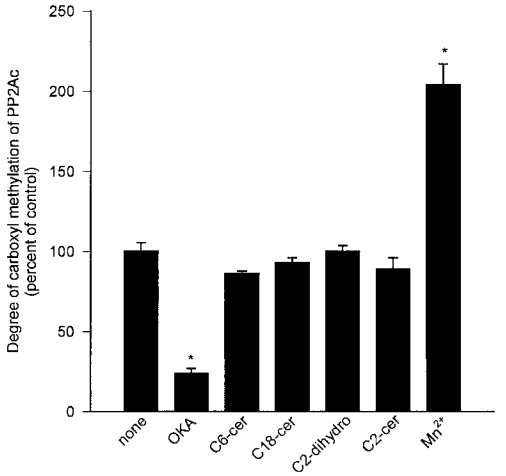

Fig. 4. Lack of effect of ceramides on the carboxyl methylation of PP2Ac. Carboxyl methylation of PP2Ac was measured in the INS cell cytosolic fraction at 37°C for 30 min using [^{3}H]*S*-adenosyl methionine in the presence of various ceramides (12.5 μM). For comparative purposes, effects of OKA (50 nM) and Mn^{2+} (5 mM) were also tested in this experiment [13]. Data are means ± S.E.M. of 3–5 determinations in each case. These data indicate that ceramides exerted no significant effects on the carboxyl methylation of PP2Ac. In agreement with data described by us recently, OKA [13] and Mn^{2+} [19] significantly inhibited and stimulated the carboxyl methylation of PP2Ac, respectively, thereby providing negative and positive internal controls.

In contrast, ceramides had no effect on OKA-insensitive phosphatase activity (Fig. 3), suggesting that the stimulatory effects of ceramides are specific to PP2A-like activity, compatible with previous reports [10,11].

Recently, we [13] and others [16,17] have presented evidence to suggest that PP2Ac undergoes an OKA-sensitive carboxyl methylation which is associated with an increase in its catalytic activity. Therefore, we investigated whether ceramide-induced increases in the catalytic activity of PP2A are due to their stimulatory effects on the carboxyl methylation of PP2Ac. None of the ceramides significantly affected the carboxyl methylation of PP2Ac (Fig. 4).

4. Discussion

Even though a growing body of experimental evidence indicates key regulatory roles for ceramides in cellular function in several cell types, very little is known about their involvement in pancreatic β cell function and metabolism. Recently, Sjöholm reported [9] that exposure of rat islets to ceramides resulted in significant reduction in insulin production and mitogenesis.

These effects appeared to be superimposable to those observed in the presence of IL-1β, suggesting that ceramides might play a role in cytokine-derived signalling in β cells. Major and coworkers [10] have presented evidence, in abstract form, that long-term (24–96 h) exposure of isolated β TC3 cells to C2- or C6-ceramides (but not inactive dihydroceramide) caused marked reduction in cell viability and attenuated glucose- and carbachol-induced insulin release. More recent studies [18] from this laboratory also suggested marked reduction in sphingomyelin content in β cells exposed to cytokines (e.g. TNF, IL-1β and IFN-γ). These data may indicate that cytokine binding results in receptor activation facilitating the hydrolysis of sphingomyelin to ceramides. Together, findings of Sjöholm [9] and Major and Wolf [10,18] suggest important roles for ceramides in the cascade of events leading to decreased viability and apoptotic demise of β cells. In contrast to these findings, however, recent studies of Kwon et al. [8] failed to observe clear stimulatory effects of IL-1β or TNFα on sphingomyelin hydrolysis in normal rat islets, mouse islets and clonal RINm5F cells. These investigators concluded that sphingomyelin hydrolysis and the consequent generation of ceramides may not be necessary for IL-1β-mediated production of nitric oxide in β cells.

Data from the present study identify a PP2A-like effector protein for ceramides in the β cell, indicating that a CAPP-like activity is localized in β cells. CAPP was first reported by Dobrowsky and Hannun in T9 glioma cell cytosolic fraction [11]. In these studies ceramides stimulated, in a concentration-dependent fashion, the hydrolysis of [^{32}P]histone. CAPP effects seem to be specific toward phosphoserines and phosphothreonines, and are sensitive to OKA and insensitive to divalent metals. Based upon these findings, it was suggested that CAPP is akin to PP2A.

As indicated above, long-term exposure of β cells to ceramides causes apoptotic cell death [10,18]. Interestingly, the putative role(s) of CAPP in mediating biological effects of ceramides gained support from recent studies of Wolff et al. [7] indicating that OKA reversed the effects of ceramide on apoptosis and c-*myc* down-regulation. Since our findings suggest that PP2A is one of the target enzymes for ceramides action, it will be interesting to examine whether coprovision of PP2A inhibitors (e.g. OKA) with ceramides prevents some of the demonstrated deleterious effects of ceramide on isolated β cells [9,10,18].

It is noteworthy that the data described in this study and in our earlier studies [13,19] suggest that the carboxyl methylation of PP2Ac and the PP2A catalytic activity are dissociable under certain conditions. For example, OKA as well as phosphorylated derivatives of nucleotides or hexoses inhibited both the carboxyl methylation of PP2Ac and PP2A activity [19]. On the other hand, divalent metal ions appear to stimulate the carboxyl methylation of PP2Ac without significantly affecting the catalytic activity of PP2A [13,19]. Interestingly, as shown in the current study, ceramides stimulated PP2A activity with no demonstrable effect on the carboxyl methylation of PP2Ac. These studies may suggest that increase in the carboxyl methylation of PP2Ac is not by itself sufficient *or* required to increase PP2A catalytic activity. However, it should be kept in mind that PP2A activity measurements were carried out using an (exogenous) artificial substrate (i.e. ^{32}P-labelled histone); regulation of the hydrolysis by PP2A of endogenous islet phosphoproteins may be different under physiologic conditions.

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