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Ceramide Selectively Inhibits Calcium-Mediated Potentiation of β-Adrenergic-Stimulated Cyclic Nucleotide Accumulation in Rat Pinealocytes

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Interaction between sphingomyelin metabolism and cyclic nucleotide synthesis in rat pinealocytes was investigated by determining the effect of ceramide on adrenergic-stimulated cAMP and cGMP accumulation. Although C2-, C6-, and C8-ceramide had no effect on basal, isoproterenol-, or norepinephrine-stimulated cAMP and cGMP accumulation, they inhibited the potentiation caused by depolarising concentrations of K^+ or BayK 8644. Similar inhibition was observed when ceramide metabolism was inhibited by a glucosylceramide synthase inhibitor. In contrast, the potentiation of cAMP and cGMP accumulation caused by other intracellular Ca^{2+}-elevating agents such as ionomycin or thapsigargin or by an activator of protein kinase C was not affected by ceramide. Taken together, our results suggest that ceramide selectively inhibits cyclic nucleotide synthesis when the nucleotide synthesis is potentiated by an increase in intracellular Ca^{2+} through L-type Ca^{2+} channels and that the sphingomyelin cycle probably plays an important role in the regulation of these channels.

The production of cAMP and cGMP in rat pinealocytes requires the activation of adenylyl or guanylyl cyclases by stimulation of β-adrenergic, VIP or PACAP receptors (1-4) or by direct activation of the G-protein or the cyclases (5,6); the magnitude of these responses can be substantially modulated by other signalling pathways not directly involved in the activation of the cyclases. For example, elevation of intracellular Ca^{2+} (7,8), activation of protein kinase C (9,10) and inhibition of serine/threonine phosphatases (11) or tyrosine kinases (12,13) have all been shown to potentiate basal or stimulated cyclic nucleotide responses.

Another signalling pathway which has received much attention recently is the sphingomyelin cycle (14,15). Ceramide, which is produced following sphingomyelin hydrolysis, has been shown to be associated with the regulation of intracellular enzymes such as protein kinase C (16), tyrosine kinases (17), diacylglycerol kinase (18), phosphatases (19) and phospholipases (20). In addition, ceramide also has an effect on nuclear transcription events and apoptosis (15,21). Since both kinases and phosphatases can modulate the cyclic nucleotide responses in the rat pinealocyte, the purpose of the present investigation is to determine whether ceramide has an effect on adrenergic-stimulated cAMP and cGMP accumulation.

MATERIALS AND METHODS

Materials. Isobutylmethylxanthine (IBMX), 4β-phorbol 12-myristate 13-acetate (PMA), norepinephrine (NE), L-isoproterenol (ISO), cultured medium and fetal bovine serum were obtained from Sigma Chemical Corp. (St. Louis, MO). C2-ceramide, C6-ceramide, C8-ceramide, C2-dihydroceramide, 1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol (PPMP), genistein, thapsigargin, ionomycin, BayK 8644 and calyculin A were obtained from Calbiochem Corp. (La Jolla, CA). ^{125}I cAMP and cGMP were obtained from ICN Immunobiologicals (Lisle, IL). All other chemicals were of the purest grades available and were obtained commercially. Antibodies for the radioimmunoassays of cAMP and cGMP were gifts from Dr. A. Baukal (National Institute of Child Health and Human Development, NIH, Bethesda, MD).

Preparation and treatment of rat pinealocytes. Pinealocytes were prepared from male Sprague-Dawley rats (150 gm, University of Alberta Animal unit) by trypsinization as previously described (3, 22). The cells were suspended in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and maintained at 37°C for 24 hr in a gas mixture of 95% air and 5% CO_2 before experimental treatment.

Aliquots of cells (1.5×10^4 cells/0.4 ml) were treated with drugs which had been prepared in concentrated solutions in water or di-

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Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; IBMX, isobutylmethyl-xanthine; ISO, isoproterenol; NE, norepinephrine; PMA, 4β-phorbol 12-myristate 13-acetate; PPMP, 1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol.

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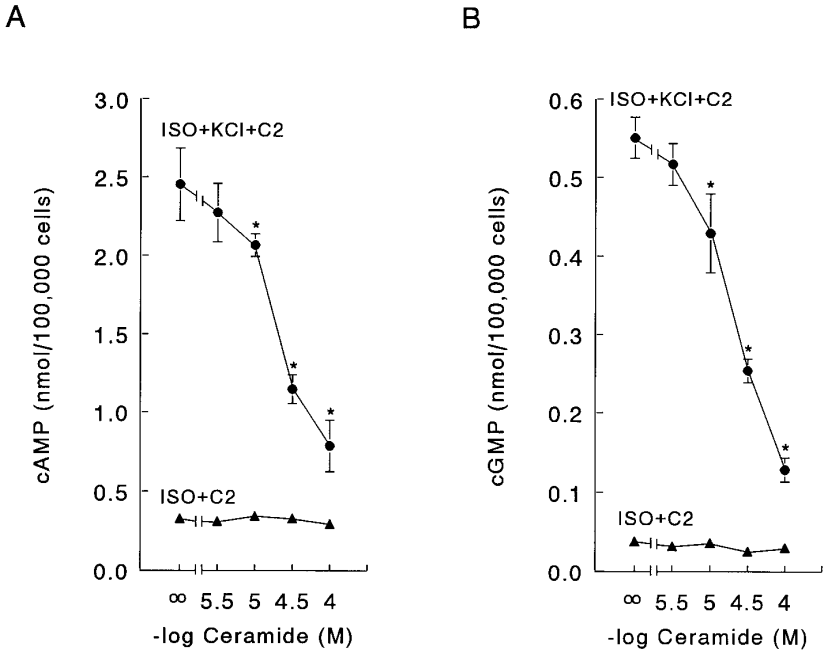

FIG. 1. Effect of C2-ceramide on ISO- and ISO+KCl-stimulated cAMP and cGMP accumulation in rat pinealocytes. Pinealocytes (1.5×10^4 cells/ 400μl) were incubated in DMEM with 10% fetal bovine serum and pre-treated with or without C2-ceramide (3-100μM) for 5 min. The cells were then stimulated with ISO (1μM) in the presence or absence of KCl (30mM) for an additional 15 min. Each value represents the mean ± SEM of determinations done in duplicate on three samples of cells. For further details, see Materials and Methods. *Significantly different from treatment with ISO+KCl.

methylsulfoxide. The final concentration of the latter never exceeded 0.5%. At this concentration, dimethylsulfoxide has no effect on the NE- or ISO-stimulated cAMP and cGMP responses. The duration of the drug treatment period was 15 min for cAMP and cGMP accumulation unless indicated otherwise. At the end of the treatment period, cells were collected by centrifugation (2 min, 10,000g), the supernatant was aspirated, and the tube was placed on solid CO_2. The frozen cell pellets were lysed by the addition of 5 mM acetic acid (100 μl) and boiling (5 min). The lysates were stored frozen at −20°C until analysis.

Cyclic nucleotide assays. The lysates were centrifuged (10 min, 12,000g) and samples of the supernatant were used to determine cellular cAMP and cGMP content, using a radioimmunoassay procedure in which samples were acetylated prior to analysis (3,23). Since there was a small batch-to-batch variation of the cyclic nucleotide responses between cell preparations, all comparisons were performed within the same batch of cells.

Statistical analysis. Data are presented as the mean ± SEM of the amount of cAMP and cGMP in three aliquots of cells. Each experiment was repeated at least twice with different cell preparations. The amount of cyclic nucleotide in each cell pellet was based on duplicate determinations. Data were analyzed by Duncan's multiple range test (24), with statistical significance set at $P < 0.05$.

RESULTS

Effects of Ceramides on ISO and ISO + KCl-Stimulated Cyclic Nucleotide Accumulation

Treatment with ISO (1μM) alone caused a significant increase in cAMP and cGMP accumulation; the addition of a depolarising concentration of K^+ (30 mM KCl) potentiated these responses by 8-10 fold (Fig. 1). C2-ceramide had no effect on basal cAMP and cGMP accumulation (data not shown). Pretreatment with C2-ceramide for 5 min had no effect on ISO-stimulated cAMP or cGMP accumulation, but inhibited the ISO+KCl-stimulated responses in a dose-dependent manner. Significant inhibition was observed at 10μM of C2-ceramide and at 100μM the reduction was more than 70% for both cAMP and cGMP responses. C6 and C8-ceramides were also effective in reducing the ISO+KCl-stimulated cAMP and cGMP responses. In contrast, C2-dihydroceramide, an inactive analogue, had no effect (Table 1).

Effects of Ceramide on Cyclic Nucleotide Accumulation Stimulated by ISO in the Presence of Other Potentiating Agents

The purpose of these experiments was to determine whether the inhibitory effect of C2-ceramide is dependent on the mechanism of potentiation. In accordance with previous reports (7-13), co-treatment with phenylephrine (an α-adrenergic agonist), PMA (a protein kinase C activator), genistein (a tyrosine kinase inhibitor), calyculin A (a serine/threonine phosphatase inhibitor) or IBMX (a phosphodiesterase inhibitor) potentiated the ISO-stimulated cyclic nucleotide responses (Table 2, Experiment I). Pre-treatment with C2-ceramide for 15 min had no effect on these potentiating agents (Table 2, Experiment I). These results suggest that the inhibitory

TABLE 1

Effects of Ceramides on the Potentiating Effect of K^+ on ISO-Stimulated cAMP and cGMP Responses

Treatment	cAMP (nmol/10^5 cells)	cGMP (nmol/10^5 cells)
Control	0.05 ± 0.01	0.008 ± 0.002
ISO (1 μM)	0.33 ± 0.03	0.038 ± 0.003
ISO (1 μM) + KCl (30 mM)	2.24 ± 0.27	0.498 ± 0.033
+ C2-ceramide (30 μM)	1.43 ± 0.12*	0.312 ± 0.029*
+ C6-ceramide (30 μM)	1.15 ± 0.09*	0.254 ± 0.015*
+ C8-ceramide (30 μM)	1.64 ± 0.14*	0.376 ± 0.023*
+ C2-dihydroceramide (30 μM)	2.14 ± 0.13	0.457 ± 0.041

Note. Pinealocytes (1.5×10^4 cells/400 μl) were incubated in DMEM with 10% fetal bovine serum and pre-treated with or without ceramides (30 μM) for 5 min. The cells were then stimulated by ISO (1 μM) and KCl (30 mM) for an additional 15 min. Each value represents the mean ± SEM of determinations done in duplicate on three samples of cells.

* Significantly different from treatment with ISO+KCL.

effect of C2-ceramide may be specific to intracellular Ca^{2+}-elevating agents.

To investigate further whether the inhibitory effect of C2-ceramide is dependent on the mechanism by which intracellular Ca^{2+} is elevated, different intracellular Ca^{2+}-elevating agents were used. As shown in Table 2 (Experiment II), ionomycin (a Ca^{2+} ionophore), BayK 8644 (an L-type Ca^{2+} channel agonist) and thapsigargin (an intracellular Ca^{2+}-ATPase inhibitor) were all effective in potentiating the ISO-stimulated cAMP and cGMP responses. Pre-treatment with C2-ceramide for 5 min inhibited only the BayK 8644-mediated potentiation of the cAMP and cGMP responses (Table 2, Experiment II).

Effects of PPMP on ISO-, ISO + K^+- and ISO+BayK 8644-Stimulated Cyclic Nucleotide Accumulation

Treatment with a glucosylceramide synthase inhibitor, PPMP, has been shown to induce an increase in cellular ceramide levels (24,25). Pre-treatment with PPMP for 15 min had an effect similar to that of C2-ceramide: PPMP had no effect on basal or ISO-stimulated cAMP and cGMP accumulation, but it inhibited the ISO+KCl- or ISO+BayK 8644-stimulated cAMP and cGMP accumulation (Table 3). In contrast, PPMP had no effect on the potentiation induced by ionomycin, PMA, calyculin A, genistein or thapsigargin (Table 3).

DISCUSSION

Our previous studies have indicated that cyclic nucleotide accumulation in the rat pinealocyte is subject to modulation by multiple signalling mechanisms (6-13). The present study examined whether ceramide, a second messenger from the sphingomyelin cycle, also modulates cyclic nucleotide synthesis in the rat pineal gland. Our results indicate that although ceramide on its own does not have a direct effect on cyclic nucleotide synthesis, it significantly alters the modulating effect of some signalling pathways.

We have previously established that pineal cyclic nucleotide synthesis can be potentiated by agents that acti-

TABLE 2

Effects of Ceramides on the cAMP and cGMP Responses Stimulated by ISO in the Absence or Presence of Potentiating Agents

Treatment	cAMP (nmol/10^5 cells)		cGMP (nmol/10^5 cells)	
	−C2	+C2	−C2	+C2
Experiment I				
ISO (1 μM)	0.33 ± 0.04	0.38 ± 0.06	0.04 ± 0.01	0.04 ± 0.01
ISO (1 μM) + KCl (30 mM)	2.34 ± 0.32	1.03 ± 0.11*	0.48 ± 0.02	0.19 ± 0.02*
ISO (1 μM) + PMA (0.1 μM)	3.54 ± 0.23	3.34 ± 0.17	0.07 ± 0.01	0.06 ± 0.01
ISO (1 μM) + genistein (30 μM)	0.65 ± 0.03	0.67 ± 0.04	0.22 ± 0.02	0.22 ± 0.03
ISO (1 μM) + calyculin A (1 μM)	2.83 ± 0.09	2.72 ± 0.14	0.13 ± 0.03	0.11 ± 0.02
ISO (1 μM) + IBMX (1 mM)	1.69 ± 0.09	1.54 ± 0.12	0.33 ± 0.03	0.30 ± 0.02
Experiment II				
ISO (1 μM)	0.32 ± 0.02	0.28 ± 0.03	0.04 ± 0.01	0.05 ± 0.01
ISO (1 μM) + BayK 8644 (1 μM)	1.96 ± 0.21	0.76 ± 0.03*	0.43 ± 0.02	0.15 ± 0.03*
ISO (1 μM) + ionomycin (10 μM)	2.28 ± 0.14	2.14 ± 0.31	0.57 ± 0.01	0.54 ± 0.03
ISO (1 μM) + thapsigargin (1 μM)	0.94 ± 0.04	0.92 ± 0.07	0.31 ± 0.03	0.28 ± 0.03

Note. Pinealocytes (1.5×10^4 cells/400 μl) were incubated in DMEM with 10% fetal bovine serum and pre-treated with (+) or without (−) C2-ceramide (C2, 30 μM) for 5 min. The cells were then stimulated by ISO (1 μM) in the presence and absence of potentiating agents, as indicated, for an additional 15 min. Each value represents the mean ± SEM of determinations done in duplicate on three samples of cells.

* Significantly different from corresponding treatment without C2-ceramide.

TABLE 3

Effect of PPMP on the cAMP and cGMP Responses Stimulated by ISO in the Absence or Presence of Potentiating Agents

Treatment	cAMP (nmol/10^5 cells)		cGMP (nmol/10^5 cells)	
	−PPMP	+PPMP	−PPMP	+PPMP
ISO (1 μM)	0.30 ± 0.01	0.28 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
ISO (1 μM) + KCl (30 mM)	2.37 ± 0.21	0.93 ± 0.14*	0.53 ± 0.03	0.22 ± 0.02*
ISO (1 μM) + BayK 8644 (1 μM)	2.03 ± 0.16	0.81 ± 0.08*	0.42 ± 0.03	0.18 ± 0.01*
ISO (1 μM) + ionomycin (10 μM)	2.43 ± 0.19	2.55 ± 0.21	0.62 ± 0.02	0.60 ± 0.04
ISO (1 μM) + thapsigargin (1 μM)	1.01 ± 0.07	0.98 ± 0.05	0.32 ± 0.02	0.29 ± 0.03
ISO (1 μM) + PMA (0.1 μM)	3.16 ± 0.18	3.24 ± 0.19	0.08 ± 0.01	0.06 ± 0.01
ISO (1 μM) + genistein (30 μM)	0.71 ± 0.04	0.70 ± 0.03	0.20 ± 0.01	0.23 ± 0.02
ISO (1 μM) + calyculin A (1 μM)	2.46 ± 0.12	2.28 ± 0.17	0.16 ± 0.02	0.14 ± 0.01

Note. Pinealocytes (1.5×10^4 cells/400 μl) were incubated in DMEM with 10% fetal bovine serum and pre-treated with (+) or without (−) PPMP (10 μM) for 15 min. The cells were then stimulated with ISO (1 μM) in the presence or absence of potentiating agents, as indicated, for an additional 15 min. Each value represents the mean ± SEM of determinations done in duplicate on three samples of cells.
* Significantly different from corresponding treatment without PPMP.

vate PKC, inhibit tyrosine kinases, elevate intracellular Ca^{2+} or inhibit serine/threonine phosphatases (7-13). Ceramide appears to inhibit selectively the potentiation mediated by elevation of intracellular Ca^{2+}; it has no effect on the potentiation mediated by PMA, genistein or calyculin A. Furthermore, this inhibitory effect of ceramide is highly specific and depends on the mechanism through which intracellular Ca^{2+} is elevated. For example, ceramide inhibits only the potentiation caused by agents such as depolarizing concentrations of K^+ or BayK 8644 which elevate intracellular Ca^{2+} through activation of the L-type Ca^{2+} channel. Ceramide has no effect on the potentiation mediated by ionomycin or thapsigargin, two agents which elevate intracellular Ca^{2+} in rat pinealocytes (8). Our results therefore suggest that an increase in ceramide levels significantly inhibits L-type Ca^{2+} channels in rat pinealocytes.

The inhibitory effect of ceramide on the nucleotides is unlikely to be due to a non-specific action because 1) C2-, C6- and C8-ceramide produce similar effects on the nucleotides; 2) C2-dihydroceramide, an inactive analogue of ceramide, is without effect; and 3) PPMP, which inhibits the metabolism of ceramide and has been shown to be effective in elevating ceramide levels in other cell types (25,26), produces the same effect as exogenous ceramide. Indeed, our data on PPMP indicate that there may be a high rate of turnover of sphingomyelin in the rat pineal gland since 15 min of pretreatment with PPMP is sufficient to generate enough ceramide endogenously to produce a similar inhibitory effect to that observed after exogenous ceramide.

The observation that ceramide inhibits only the potentiation induced by agents that elevate intracellular Ca^{2+} through activation of the L-type Ca^{2+} channel is of interest. Our results, together with the known involvement of ceramide in the inhibitory effect of cytokines on the L-type Ca^{2+} channel current in rat ventricular myocytes (27), suggest that the sphingomyelin cycle likely plays an important role in the regulation of L-type Ca^{2+} channels in rat pinealocytes. Although the endogenous activator of the sphingomyelin cycle in the pineal remains unknown, possible candidates include interleukin-1, γ-interferon, tumor necrosis factor-α, and 1,25$(OH)_2$ -vitamin D_3 (15). It will be of interest to determine whether these compounds have an effect on the L-type Ca^{2+} channel in the rat pineal gland.

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