

Differential effects of ceramides upon adenylyl cyclase subtypes

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Abstract Ceramides are reported to stimulate different effector systems, among them atypical protein kinases C (PKCs). When HEK 293 cells, stably expressing adenylyl cyclase type II (AC II), were treated with various ceramide derivatives, adenylyl cyclase activity was enhanced 8–15-fold. The stimulation by the most potent analog, C₁₈/C₂₄ ceramide, was comparable to that by the phorbol ester TPA. The stimulatory effect of ceramide was not restricted to AC II, although the type I and type V enzymes were affected less dramatically. Unexpectedly, the dihydro derivatives of ceramides, generally serving as non-activating controls, exhibited only slightly lower stimulation than ceramides, whereas short-chain ceramides (e.g. C₂) were without effect. The action of ceramides was at least partially inhibited by okadaic acid, suggesting involvement of a phosphatase. Furthermore, ceramides and TPA operated synergistically. While the PKC inhibitor staurosporine counteracted the action of phorbol esters, it significantly (2.5×) enhanced the effect of ceramides.

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Key words: Adenylyl cyclase type II; Ceramide; Phorbol ester; Okadaic acid

1. Introduction

Adenylyl cyclases from higher organisms are regulated by phosphorylation as a consequence of crosstalk with other signaling pathways [1–5]. These generate second messengers which stimulate protein kinase C (PKC). While the second messenger diacylglycerol activates classical and novel PKC subforms, the recently emerged second messenger ceramide is one of the stimulators of atypical PKCs. Ceramide is generated from sphingomyelin (e.g. via TNF-α), which through activation of a cytosolic phospholipase A (cPLA) and liberation of arachidonic acid [6] regulates neutral or acidic sphingomyelinases (Smases). The latter in turn release ceramide from the plasma membrane or from intracellular compartments. The targets of ceramide have not been fully elucidated, but among them are a membrane-associated protein kinase, a cytosolic protein phosphatase 2A and the ζ-isoform of PKC [7–9].

We have recently shown that adenylyl cyclase type II (AC II) is a preferred target of phorbol ester-stimulated PKC and have presented evidence by site-directed mutagenesis that Thr-1057 is the phosphoryl acceptor particularly involved in sensitization via phorbol ester-stimulated PKCs. However, stimulation by ceramide as a potential activator of atypical PKCs was not affected by this mutation [10,11].

Here we show that other adenylyl cyclase subtypes besides AC II, namely AC I and AC V, stably expressed in HEK 293 cells, are also directly or indirectly targets of a ceramide-dependent event, leading to enhanced enzymatic activity. Since

dihydroceramides were qualitatively similarly effective, the underlying mechanism seems to differ from the classical ceramide action. The site of the ceramide-induced modification, resulting in activation of the catalyst, is not known yet. However, the fact that the effect was prevented by okadaic acid and enhanced by staurosporine argues for a phosphatase being involved.

2. Materials and methods

2.1. Materials

C₁₈/C₂₄ ceramide (type III), C₁₆ dihydroceramide, TPA, isobutylmethylxanthine, adenosine-5'-triphosphate were obtained from Sigma. C₂ ceramide and C₂ dihydroceramide were from Biomol. Fetal calf serum, 5'-cyclic AMP, DNase I, DOSPER and alkaline phosphatase (molecular biology grade) were purchased from Boehringer (Mannheim). Minimum essential medium (MEM) and G-418 were from Gibco and Boehringer, Ingelheim respectively. [α-³²P]ATP, [³H]adenine and okadaic acid were obtained from ICN; C₁₆ ceramide and staurosporine were purchased from Calbiochem.

2.2. Stable transfection of HEK 293 cells

HEK 293 cells were grown at 37°C in MEM supplemented with 10% fetal bovine serum in a humidified 95% air/5% CO₂ incubator.

2.2.1. AC I. A HindIII-XbaI fragment from bovine brain type I adenylyl cyclase [12] was subcloned from pSK-ACI into SmaI-restricted pXMD1 [13] via blunt-end ligation. Thereafter the type I cDNA was excised from pXMD-ACI_{rev} with EcoRI and SalI, inserted into EcoRI-XhoI-restricted pcDNA3 and used for transfection of HEK 293 cells by the calcium phosphate method. Neomycin-resistant cells were selected in culture medium containing G-418 (500 µg/ml).

2.2.2. AC II. The full length cDNA for rat type II adenylyl cyclase [14] was excised from pBluescript with EcoRI, introduced into pXMD1 and, together with pRSV neo, used for transfection of HEK 293 cells by the calcium phosphate method. Neomycin-resistant cells were selected in culture medium containing G-418 (400 µg/ml).

2.2.3. AC V. The full length cDNA for type V adenylyl cyclase from rabbit myocardium [15] was excised from pXMD-AC V with KpnI and XhoI, introduced into pcDNA3 and used for transfection of HEK 293 cells by lipofection with DOSPER. Neomycin-resistant cells were selected in culture medium containing G-418 (500 µg/ml).

2.3. Measurement of AC activity following [³H]adenine labeling

The assay was performed according to Salomon [16] and Avidor-Reiss et al. [17]. Stably transfected HEK 293 cells (2.2 × 10⁵) were first incubated for 5 h at 37°C with ceramide and dihydroceramide derivatives or vehicle (see figure legends) in a total volume of 500 µl and treated with the phosphodiesterase inhibitor 1-methyl-3-isobutylxanthine (1 mM) for 10 min. The cells were then loaded with 2 µCi [³H]adenine for 60 min, followed by the addition of adenylyl cyclase stimulators. The reaction was terminated with 1/6 volume of 30% TCA, 10 mM cAMP, 10 mM ATP, 5000 cpm [³²P]cAMP and 5000 cpm [³²P]ATP as internal standards.

Adenylyl cyclase activity was calculated according to Salomon [16] as follows:

$$\% \text{ conversion} = \frac{\text{cAMP}_{\text{built}} \times 100}{(\text{cAMP}_{\text{built}} + \text{ATP}_{\text{built}})}$$

2.4. Membrane preparation

Cells were lysed by freeze/thaw in 10 mM Tris buffer, pH 7.4, 1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 3.2 µg/ml TPCK, 22 µg/ml

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TLCK, 3.2 µg/ml soybean trypsin inhibitor, 2.8 µg/ml Trasylol, 1 mM benzamidine, 0.1 mM benzethonium chloride, 10 mM NaF and 1 mM Na₂VO₄. Membranes were prepared by differential centrifugation and stored in the above buffer supplemented with 10% glycerol at –80°C.

2.5. Miscellaneous

Polyacrylamide gel electrophoresis (5–15% acrylamide) according to Laemmli [18] and immunoblotting were conducted as described previously [19]. Monoclonal antibody BBC-2, labeled with alkaline phosphatase (BBC-2-AP), was used for detection by chemiluminescence with CSPD as substrate [20]. Alkaline phosphatase was coupled to antibody according to the specifications of the distributor.

3. Results and discussion

Fig. 1 shows a time course of the naturally occurring ceramide derivative from bovine brain (a mixture of C₁₈ and C₂₄ ceramides) influencing the basal activity of AC II stably expressed in HEK 293 cells. Ceramides were suspended in aqueous buffer by dilution of a solution in ethanol/dodecane (98:2) to a final concentration of 1% following a suggestion of Li et al. [21]. Maximal stimulation was obtained after 6 h (15-fold), while prolonged treatment resulted in decline of activity, probably due to cell death.

Fig. 2 demonstrates the effect of C₁₈/C₂₄ ceramide on several representatives of adenylyl cyclase subtype families stably expressed in HEK 293 cells: AC I (Ca²⁺/CaM-stimulated, inhibited by βγ subunits), AC II (conditionally stimulated by βγ subunits in concert with α_s) and AC V (a member of the Ca²⁺-inhibitable subfamily) and the HEK 293 endogenous enzymes as control were tested for the lipid's effect on basal and forskolin-stimulated activity. As can be seen from the inset, all subforms were readily expressed. The largest effect of ceramide could be observed with AC II, the non-stimulated activity of which was enhanced 8–15-fold, while that of AC I and AC V was only doubled. Besides basal activity, stimulation by forskolin was also affected. Although with AC II forskolin stimulation was still three times higher than in control cells, the action of the diterpene may be more precisely seen as inhibition of non-stimulated activity. Although not inhibited by forskolin as shown for AC II, ceramide-treated HEK 293 cells expressing AC I or AC V were significantly less stimulated by forskolin (40–70%) than their untreated controls. As an example, for AC V, the ratio of activities in the

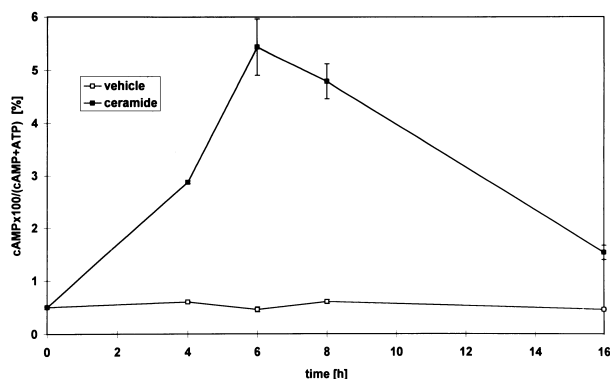


Fig. 1. Time course of C₁₈/C₂₄ ceramide-stimulated HEK 293 AC II activity. HEK 293 AC II cells (see Section 2) were incubated with C₁₈/C₂₄ ceramide (20 µM) or ethanol:dodecane (98:2) as control (final concentration 1%) for the indicated times and then labeled with [³H]adenine for 60 min. cAMP formation was measured after another 10 min as described in Section 2. Values are given as duplicates ± S.E.M.

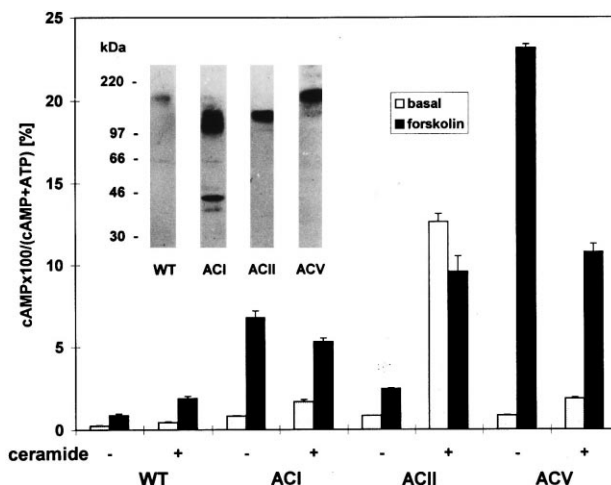


Fig. 2. Influence of C₁₈/C₂₄ ceramide on cells expressing other adenylyl cyclase subtypes. HEK 293 cells were stably transfected with cDNAs encoding adenylyl cyclase subtypes I, II and V as described in Section 2. Cells were incubated with C₁₈/C₂₄ ceramide (20 µM) or ethanol:dodecane (98:2) as control (final concentration 1%) for 5 h, labeled with [³H]adenine for 60 min and stimulated with and without forskolin (10 µM; 10 min). cAMP formation was measured as described in Section 2. Values are given as duplicates ± S.E.M. Inset: Western blot analysis of AC subtypes I, II and V. Membranes from HEK 293 cells (WT 25 µg) or from HEK 293 cells stably transfected with AC I (25 µg), AC II (50 µg) or AC V (200 µg) were separated by SDS-PAGE, blotted onto Immobilon P membrane and visualized by antibody BBC-2-AP and chemiluminescence as described in Section 2. The bands in the lower molecular weight range (AC I) are probably due to proteolysis.

presence and absence of forskolin dropped from 27.5 to 5.5 after ceramide treatment.

In contrast to stimulation by forskolin, that by 10⁻⁵ M isoproterenol was still present in cells expressing AC II previously treated with ceramide (1.6-fold, not shown). The loss of forskolin stimulation of AC II on treatment with C₁₈/C₂₄ ceramide was in contrast to that on treatment with phorbol esters as forskolin was still a potent activator in TPA-stimulated cells (see also Fig. 4). All effects of ceramide on AC II (enhanced basal activity, reduced stimulation with forskolin) were also observed with HEK 293 cells expressing the cyclase transiently, as reported previously [11]. For obvious reasons, AC II was the object of the following studies.

In Fig. 3 a dose-response curve of various ceramide derivatives and the effect on non-stimulated activity of AC II is shown. It is evident that the mixture of C₁₈ and C₂₄ ceramides was most efficient, while the C₁₆ derivative was about half as effective and the acetyl derivative was stimulating only at concentrations > 20 µM. Most remarkable, however, was the fact that the dihydro derivatives of ceramides, otherwise known as inactive controls, were almost as effective (60–80%) as their unsaturated counterparts. The following studies in this paper were conducted with the most potent C₁₈/C₂₄ ceramide derivative mixture.

Of further importance was the finding that ceramide and the phorbol ester TPA activated synergistically (Fig. 4) at least in the absence of forskolin or other cyclase stimulators.

Next we wanted to investigate whether the effect of C₁₈/C₂₄ ceramide on AC II was due to stimulation of a protein kinase C. In the absence of C₁₈/C₂₄ ceramide, the potent PKC inhibitor staurosporine led to a modest enhancement of basal and a

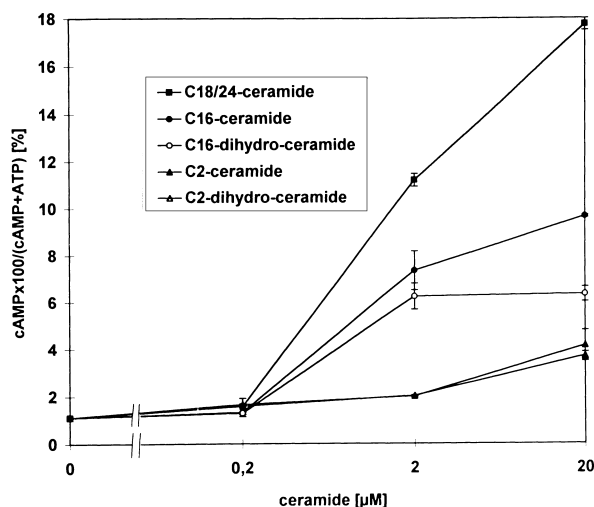


Fig. 3. Stimulation of HEK 293 AC II by different ceramide and dihydroceramide derivatives. HEK 293 AC II cells were incubated with various ceramide and dihydroceramide derivatives or ethanol:dodecane (98:2) as control (final concentration 1%) for 5 h and then labeled with [3 H]adenine for 60 min. cAMP formation was measured after another 10 min incubation as described in Section 2. Values are given as duplicates \pm S.E.M.

more pronounced enhancement (3-fold) of forskolin-stimulated activity (Fig. 5A). Surprisingly, the activity induced by ceramide was further potentiated (2.5–3.4-fold) by staurosporine in the absence or presence of 10 μ M forskolin (Fig. 5B). As a control, we tested the effect of staurosporine on TPA-induced stimulation of adenylyl cyclase activity. As was to be expected, staurosporine significantly counteracted the TPA effect both in the presence and in the absence of 10 μ M forskolin (not shown). Although it has been reported that staurosporine caused ceramide formation in neuronal cells [22], this cannot explain the synergistic interaction between staurosporine and ceramide shown here.

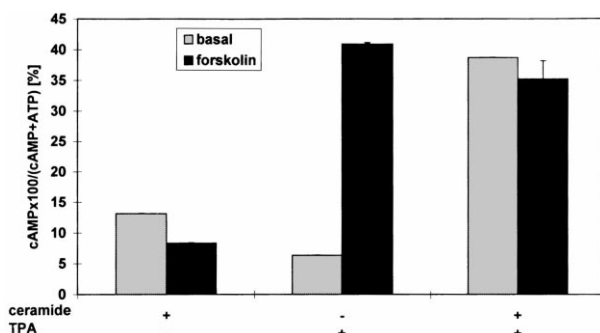


Fig. 4. Synergistic stimulation of AC II by C_{18}/C_{24} ceramide and phorbol ester. HEK 293 AC II cells were incubated with C_{18}/C_{24} ceramide (20 μ M) or TPA (1 μ M) or a combination of both. Incubation with ceramide was for 5 h, followed by [3 H]adenine labeling for 60 min and by treatment for 15 min with and without TPA. cAMP formation in the absence or presence of 10 μ M forskolin was measured after 10 min as described in Section 2. Values are given as duplicates \pm S.E.M. The vehicles for ceramide and for TPA were ethanol:dodecane (98:2) (1%) and DMSO (0.1%) respectively. They did not affect cAMP formation. Basal and forskolin-stimulated cells in the absence of ceramide or TPA showed 0.3% and 1.1% conversion respectively.

In contrast, as shown in the experiment in Fig. 6, the ceramide-induced activation was significantly inhibited by okadaic acid, known to have the highest affinity for PP-2A phosphatases. The degree of inhibition by okadaic acid was variable, ranging from 40% to 70%. Thus the influence of ceramide was at least partially explained by its activation of a protein phosphatase. As was to be expected, the activation by the phorbol ester TPA was not affected by 50 nM okadaic acid (not shown).

Treatment of HEK 293 cells, overexpressing adenylyl cyclase subtypes, with ceramides had some quite remarkable consequences especially on the type II subform. C_{16} and more so C_{18}/C_{24} ceramide led to a dramatic stimulation of adenylyl cyclase activity, comparable or even superior to that obtained with phorbol esters.

A peculiarity to be mentioned was that the dihydro derivatives of ceramides, normally known as inactive controls, were almost as effective stimulators as the ceramides themselves, and that short-chain ceramides (C_2) were considerably less potent than long-chain derivatives. Another unexpected, and as yet unexplained, finding was the significant inhibition of adenylyl cyclase by the prominent stimulator forskolin in ceramide-treated HEK 293 AC II cells. Inhibitory effects of forskolin at low concentrations have been previously observed with GTP γ S-pretreated platelet adenylyl cyclase [23] and with hCG-stimulated Leydig cell adenylyl cyclase [24]. Although in the latter system the effect was prevented by pertussis toxin,

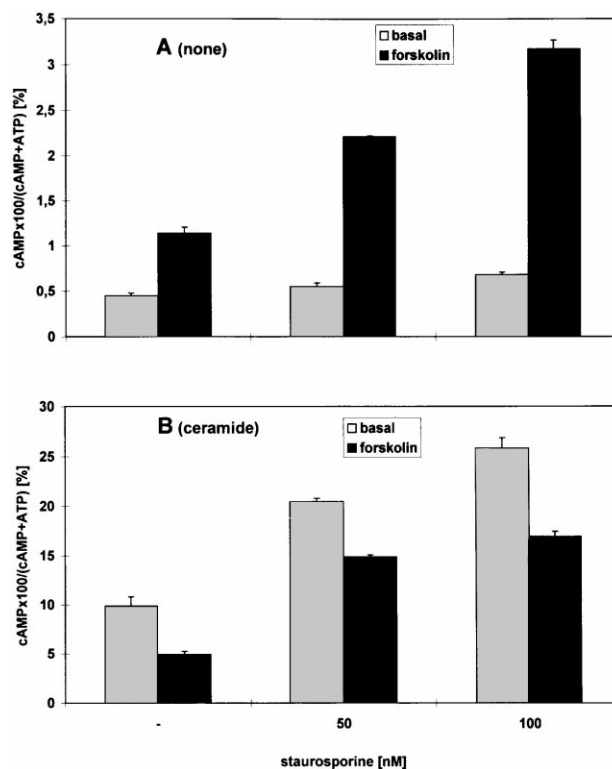


Fig. 5. Synergism between staurosporine and C_{18}/C_{24} ceramide on activation of AC II. HEK 293 AC II cells were preincubated with staurosporine or DMSO as vehicle for 1 h and then treated with ethanol:dodecane (98:2, 1%) (A) or 20 μ M C_{18}/C_{24} ceramide (B) for 5 h, followed by labeling with [3 H]adenine for 60 min. For estimation of cAMP formation (see Section 2) cells were stimulated with and without forskolin (10 min). Values are given as duplicates \pm S.E.M.

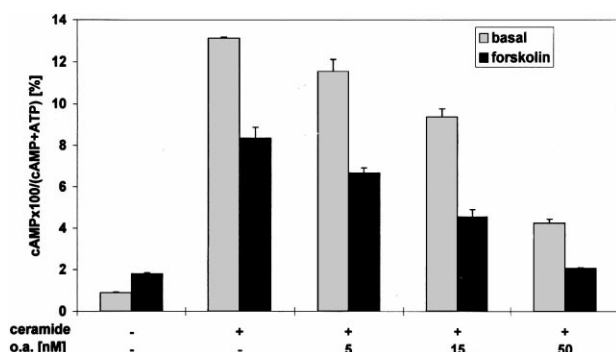


Fig. 6. Partial inhibition of C_{18}/C_{24} ceramide action on AC II by okadaic acid. HEK 293 AC II cells were preincubated with okadaic acid for 1 h. To investigate the effect of C_{18}/C_{24} ceramide, cells were treated without and with 20 μ M ceramide for 5 h, followed by labeling with [3 H]adenine. For estimation of cAMP formation (see Section 2) cells were stimulated with and without 10 μ M forskolin (10 min). Values are given as duplicates \pm S.E.M.

an explanation on a molecular basis could not be provided in either case. Effects of ceramides on other adenylyl cyclase subforms (I, V) were different. While basal activity was only marginally affected, stimulation by forskolin was significantly reduced especially in the case of AC V.

Regarding other cellular aspects, phorbol esters and ceramides normally counteract each other [25,26]. Yet, at least in the adenylyl cyclase II system described here, both interacted in a synergistic way (Fig. 4).

Further studies have to be designed to explore the exact mechanism by which exogenous ceramide may activate adenylyl cyclase, especially type II. Furthermore, in the light of the non-canonical action of ceramide it has to be decided whether ceramide itself or a metabolite is the activating species. On the other hand, the target has to be identified which is modified directly or indirectly by ceramide. There is evidence that this may not be the adenylyl cyclase itself, since the effect of ceramide, while still demonstrable in permeabilized cells, was completely lost when membranes were prepared from treated cells (A. Bösel, unpublished). Otherwise the putative modification of the catalyst may be reversible and may not withstand further fractionation of the lysed cell.

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