Ceramidase activity in porcine epidermis

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This report presents the first demonstration of a ceramide-hydrolyzing activity in mammalian epidermis. An assay using fractions derived from porcine epidermis and synthetic [^{3}H]ceramide is described, and it is shown that under the conditions used, the K_m for ceramide is 110 μM and hydrolysis is linear for up to 2 h. The enzyme activity is maximal at pH 8–9. The specific activity of ceramide hydrolase decreases as the protein concentration in the assay mixture increases, suggesting the possibility of a dissociable inhibitor. Also, the activity can be inhibited by added palmitic acid. Ceramide hydrolysis may be an important regulatory mechanism in the epidermis due to the ability of the liberated free sphingosine to modulate the activity of protein kinase C.

Ceramide; Sphingosine; Sphingolipid; Epidermis

1. INTRODUCTION

The demonstration that sphingosine and related long-chain bases can inhibit protein kinase C [1] has led to much interest in this group of amines. Thus, it has been demonstrated that exogenously supplied sphingosines can inhibit a variety of protein kinase C-mediated events in cells in culture [1–4] and in vivo [5], and it has been proposed that endogenously produced sphingosine may serve as a second messenger [4].

In mammalian epidermis, where ceramides are a major product of terminal differentiation, recent studies have revealed a much higher content of free long-chain bases [6] than found in other tissues [2,7,8]. It was proposed that this free sphingosine is produced by ceramide hydrolysis and may provide a feedback mechanism for the regulation of epidermal growth and differentiation [6]. If this is correct, then ceramide hydrolase should be demonstrable in epidermis. The present study demonstrates ceramide hydrolytic activity in fractions derived from pig epidermis.

2. MATERIALS AND METHODS

2.1. *Preparation of epidermal homogenate*

Epidermis was prepared as previously described [9] and stored frozen at −20°C. Epidermis (320 cm^2), or an equal area of stratum corneum, was pulverized under liquid nitrogen with a mortar and pestle. The ground epidermis was allowed to warm to near room temperature before addition of 20 ml of 1 mM, pH 7.4, Tris-HCl containing 1 mM EDTA, 0.25 M sucrose and 12 mM sodium deoxycholate. The mixture was ground an additional 3 min, sonicated for 1 min, and centrifugation at 1000 × g for 10 min. The supernatant was used in the ceramidase assays. Protein was measured by a modification [10] of the method of Lowry [11].

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2.2. *Synthesis of ceramide*

[9,10-^{3}H]Palmitic acid (30–60 Ci/mmol; New England Nuclear, Boston, MA) was diluted with unlabeled palmitic acid (Sigma Chemical Co., St. Louis, MO) to a specific activity of 54 472 dpm/nmol. Equimolar quantities of palmitic acid (17.9 mg), *N*-hydroxysuccinimide (8 mg) and dicyclohexylcarbodiimide (14.4 mg) were dissolved in dry ethyl acetate (0.33 ml) and allowed to react overnight [12]. Ethyl acetate (3 ml) was added, and dicyclohexylurea was removed by filtration. In a modification of the method of Ong and Brady [13], the crude product was dried under nitrogen, dissolved in freshly distilled ethyl ether and added to a 25% molar excess of sphingosine (Sigma Chemical Co.). After 18 h at room temperature, the solvent was evaporated under nitrogen, and the ceramide product was isolated by preparative silicic acid thin-layer chromatography with a mobile phase consisting of chloroform methanol acetic acid (190:9:1). The ceramide band was detected by scanning at 210 nm with a photodensitometer (model CS 930; Shimadzu Corp., Kyoto, Japan). The appropriate region of silica gel was scraped from the plate, and the ceramide was eluted with chloroform methanol (2:1). The final product, with a specific activity of 54 472 dpm/nmol, was recovered in 70% overall yield.

2.3. *Assay of ceramidase*

Ceramidase was assayed at 37°C in 0.6 ml of 40 mM buffer containing 0.21 M sucrose, 0.8 mM EDTA, 4 mg/ml sodium deoxycholate, 0.7 mg/ml Triton X-100, 0.62 mM [^{3}H]ceramide and 0.1–0.5 mg of protein. Buffers used included citric acid (pH 3), acetate (pH 4 and 5), phosphate (pH 6 and 7), Tris-HCl (pH 8) and borate (pH 9). Reaction was terminated by addition of 100 μg palmitic acid and 4 ml 10% citric and 5 ml hexane. After mixing and centrifugation, the hexane phase was transferred to a clean tube and the aqueous phase was reextracted with an additional 5 ml hexane. The combined hexane extracts were dried under nitrogen, and the residue was redissolved in 100 μl of chloroform methanol (2:1). A 4-μl aliquot was taken for scintillation counting. A 16-μl was applied to a lane on a thin-layer plate, which was then developed with hexane ethyl ether acetic acid (70:30:1). The distribution of radioactivity on

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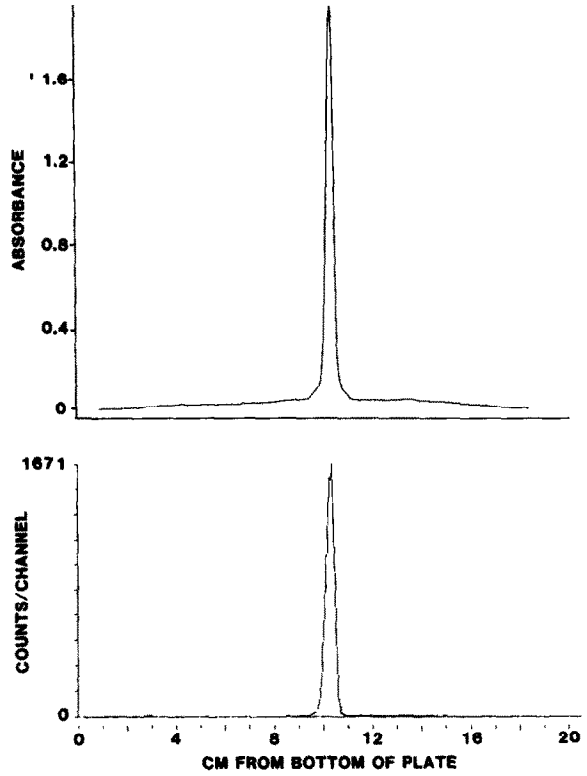

Fig. 1. Purity of synthetic [^{3}H]ceramide. The upper and lower panels show the distributions of carbon and radioactivity revealed by thin-layer chromatography of the synthetic ceramide.

the plate was determined using a linear analyzer (model LB 284; Berthold Analytical Instruments, Nashua, NH).

3. RESULTS AND DISCUSSION

Fig. 1 demonstrates the chromatographic and radiochemical purity of the synthetic ceramide substrate, the mobility of which was nearly identical to one of the previously characterized epidermal ceramides [14]. Fig. 2 shows the time-dependent release of fatty acid from this substrate by ceramidase action. Boiling destroyed this activity (not shown).

The pH profile of ceramidase activity in epidermal homogenates (Fig. 3) contains two pH optima in the ranges of 4–5 and 8–9. Although acid ceramidase is a widely distributed lysosomal enzyme [15], the alkaline ceramidase shows greater activity and persisted measureably into the stratum corneum. Therefore, all subsequent experiments were performed with epidermal homogenates at pH 9.

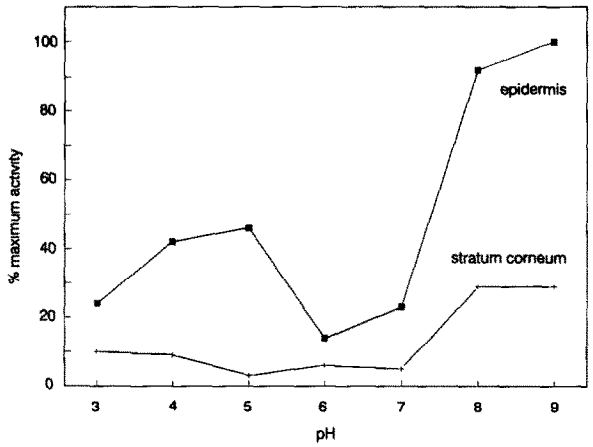

Fig. 3. pH profile of ceramidase activity.

It was determined that the time course of ceramide hydrolysis by alkaline ceramidase is linear for up to 2 h (Fig. 4) and the K_m for ceramide was estimated to be 110 1M (Fig. 5). When the concentration of protein in the assay mixture was varied, the rate of product formation per mg protein decreased as the concentration of protein increased (Fig. 6). This observation indicates an endogenous dissociable inhibitor. Free fatty acid would be a likely candidate for such an inhibitor since fatty acid has previously been shown to inhibit ceramidase [17] and is abundant in epidermis [14]. Consistent with this view is the observation that the added palmitic acid inhibits activity in a dose-dependent manner (Fig. 7). Also, the difference in ceramidase activities between homogenates of whole epidermis and stratum corneum could be accounted for by differences in free fatty acid content, since the free fatty acid content of

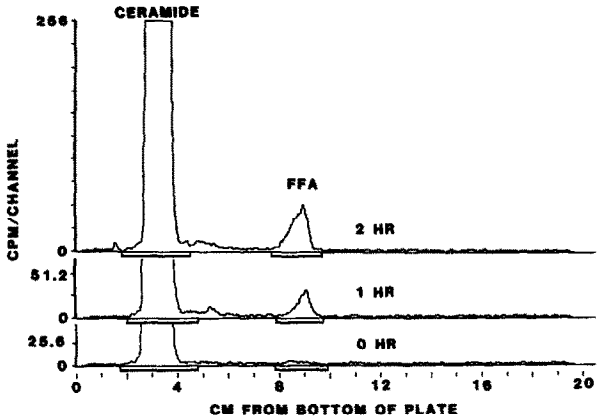

Fig. 2. Release of fatty acid from ceramide revealed by distribution of radiolabel on TLC.

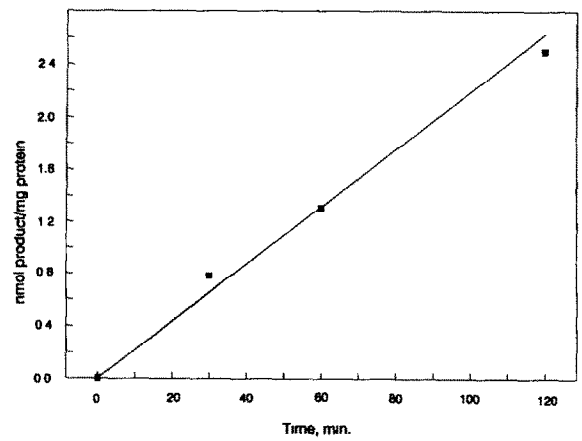

Fig. 4. Time course of ceramide hydrolysis.

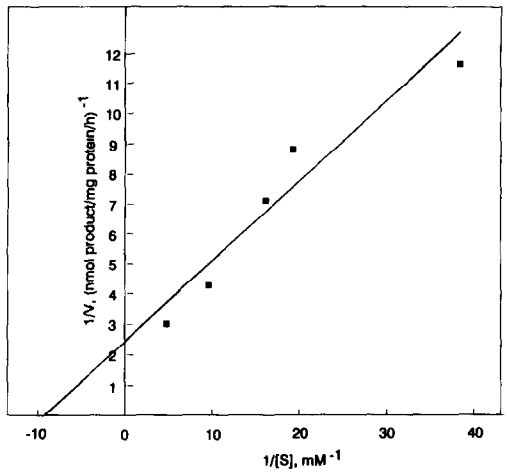

Fig. 5. $K_m = 110\ \mu M$.

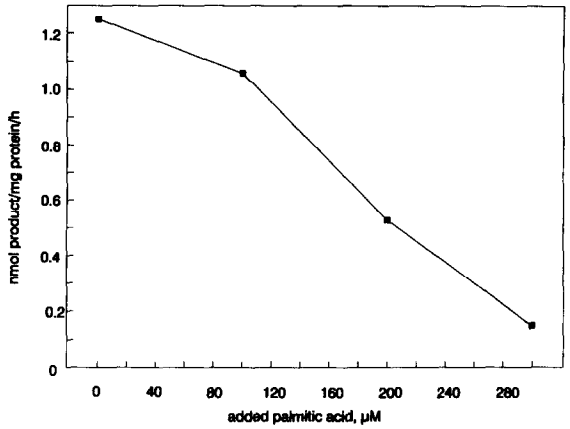

Fig. 7. Inhibition of ceramidase by added palmitic acid.

the epidermal and stratum corneum homogenates was found to be 85 and 265 μg/ml, respectively.

Ceramidases have previously been identified in a variety of tissues [15–17], but this is the first report of ceramidase in epidermis. Ceramides are present in trace amounts in most tissues where they are intermediates in the synthesis and degradation of cerebrosides, sphingomyelins and higher glycosphingolipids. In epidermis, however, ceramides themselves are the principal differentiation products, making up 40% of the lipid in stratum corneum [14], and are the major constituents of the epidermal barrier to water loss [14]. Because the ceramides are terminal differentiation products of epidermis, it would seem that ceramide hydrolysis to liberate free sphingosine could provide a feedback mechanism for regulating differentiation and ceramide synthesis.

In a previous study, it was demonstrated that the stratum corneum contains a higher concentration of free sphingosines than the viable cells [6]. The action of ceramidase to liberate free long-chain bases either within the stratum corneum or at the boundary between the living and cornified cells may provide a mechanism for communication between the terminally differentiated epidermal compartment and the differentiating and replicating compartments.

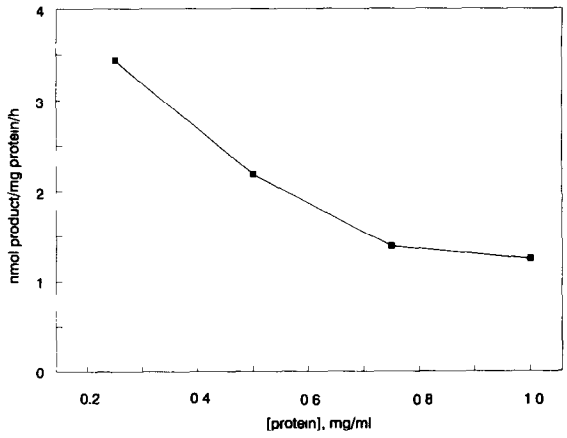

Fig. 6. Specific activity of ceramidase as a function of protein concentration.

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