Ganglioside G_{Q1b}-induced terminal differentiation in cultured mouse keratinocytes

Phosphoinositide turnover forms the onset signal

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Investigations were undertaken to see whether mouse keratinocyte differentiation was elicited by gangliosides. Among the gangliosides tested only G_{Q1b}, a tetrasialoganglioside containing two disialosyl residues, induced keratinocyte differentiation, as indicated by the formation of cornified envelopes, enhancement of transglutaminase activity and suppression of DNA synthesis. Upon stimulation with G_{Q1b} the mass content of Ins(1,4,5)P₃ and the intracellular Ca²⁺ levels were markedly enhanced in a time- and dose-dependent manner, whereas no significant changes were observed with other gangliosides, thereby indicating activation of phospholipase C for the onset of keratinocyte differentiation. Furthermore, only G_{Q1b} promoted the translocation of protein kinase C (PKC) from cytosol to membrane. Inhibition of PKC with H-7 or down-regulation of the enzyme by prolonged pre-treatment with phorbol 12,13-dibutyrate greatly suppressed transglutaminase activity and formation of cornified envelopes induced by G_{Q1b}. These results demonstrate that the tetrasialoganglioside G_{Q1b} generates the initial differentiation signal in mouse keratinocytes through phosphoinositide turnover, and also suggest that PKC activation may act at certain, as yet unidentified, stages of differentiation processes.

INTRODUCTION

Epidermal keratinocytes are programmed to differentiate terminally from basal cells into cornified cells through development of keratin filaments [1,2], formation of a cornified envelope [3] and destruction of the cell nucleus. Some important aspects of epidermal differentiation have recently been reviewed by Fuchs [4]. Work to date has documented differentiative effects of Ca²⁺ [5–7], tumour-promoting agents [8,9], and 1 α ,25-dihydroxyvitamin D₃ [1 α ,25(OH)₂D₃] [10–12]. The Ca²⁺-mediated regulation of keratinocyte differentiation has been investigated in detail [4]. The addition of 1 α ,25(OH)₂D₃ to cultured mouse keratinocytes was shown to enhance intracellular phosphoinositide hydrolysis [13]. It was therefore suggested that the increased Ins(1,4,5)P₃ mobilizes intracellular Ca²⁺ in keratinocytes, thereby providing the initial signal which, alone or in concert with other modulators, triggers differentiation. We also have investigated some biochemical events in the signal transduction system which would be involved in the onset of vitamin D₃-mediated differentiation of murine keratinocytes [14]. Our results indicated that activation of protein kinase C (PKC) is necessary, but is not the primary event in 1 α ,25(OH)₂D₃-induced terminal differentiation of the cells.

Some investigators have reported that gangliosides induce differentiation of several types of neural cells [15], modulation of transport in epithelial cells [16] and modulation of cell proliferation [17]. However, the molecular mechanisms for the expression of these functions are poorly understood. Some exogenous gangliosides can modulate protein kinase activities [18–20]. Tsuji *et al.* [21] showed that exogenously added tetrasialoganglioside G_{Q1b} promoted neurite outgrowth in human neuroblastoma cell lines. It was also demonstrated that, of various gangliosides tested, only G_{Q1b} had the ability to induce differentiation.

These findings, together with our previous work on the 1 α ,25(OH)₂D₃-induced differentiation of keratinocytes [14], prompted us to examine whether gangliosides elicit terminal differentiation of cultured mouse keratinocytes. This is the first report to demonstrate that G_{Q1b}, a tetrasialoganglioside containing two disialosyl residues, induces keratinocyte differentiation. In addition, it has been shown that the acute elevation in the intracellular Ca²⁺ concentration ([Ca²⁺]_i) mediated by phosphoinositide turnover is an essential trigger for the onset of a variety of differentiation processes.

EXPERIMENTAL

Materials

Gangliosides were purchased from Dia-Iatron (Tokyo, Japan). Fura-2/AM was obtained from Dojin Laboratories (Kumamoto, Japan). Ins(1,4,5)P₃ assay kits, [¹⁴C]putrescine, [³²P]ATP and [³H]thymidine were purchased from Amersham (Amersham, Bucks., U.K.). Monoclonal antibodies against PKC isoforms were obtained from MBL (Nagoya, Japan). All other chemicals were of reagent grade.

Cell cultures

Keratinocytes were prepared from newborn Balb/c mice using a slight modification of the procedure described by Yuspa & Harris [22]. The resulting cell suspension was filtered and the cells were plated in 60 mm dishes at an initial cell density of (1.0–1.5) × 10⁶ cells/plate in low-calcium (25 μ M-Ca²⁺) modified Eagle's medium (L-MEM) containing 5.0 ng of epidermal growth factor (EGF)/ml (Sigma Chemical Co., St. Louis, MO, U.S.A.), 180 μ g of dialysed bovine pituitary extract (BPE)/ml (Pel Freeze Co., Rogers, AR, U.S.A.) and ITS-premix (5 μ g of insulin/ml, 5 μ g of transferrin/ml and 5 ng of selenium/ml) (Collaborative

Abbreviations used: PKC, protein kinase C; PdBu, phorbol 12,13-dibutyrate; H-7, 1-(5-isoquinoline sulphonyl)-2-methylpiperazine dihydrochloride; [Ca²⁺]_i, intracellular Ca²⁺ concentration; L-MEM, low-Ca²⁺-containing modified Eagle's medium; BPE, bovine pituitary extract; EGF, epidermal growth factor; DTT, dithiothreitol; 1 α ,25(OH)₂D₃, 1 α ,25-dihydroxyvitamin D₃; ME, 2-mercaptoethanol; PBS, phosphate-buffered saline.

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Research Inc., Bedford, MA, U.S.A.) These cells were then cultivated at 37 °C in air/CO₂ (19:1).

Measurement of mass content of Ins(1,4,5)P₃

For quantitative Ins(1,4,5)P₃ assays, mouse keratinocytes were seeded in 24-well culture trays at a density of (4–8) × 10⁴ cells/ml and cultured for 24–48 h. The medium was aspirated, L-MEM containing 10 mM-LiCl was added to each well and the culture trays were allowed to re-equilibrate in a 5 % CO₂/37 °C incubator for 10 min before exposure to gangliosides. The stimulation was terminated at various times by adding 10 % (w/v) HClO₄ and the mixtures were kept on ice for 15 min. After neutralization with ice-cold 1.5 M-KOH for 60 min on ice, the samples were centrifuged at 2000 g for 10 min to remove the KClO₄ precipitate. The supernatant (100 µl) from each sample was used for Ins(1,4,5)P₃ measurement using the Ins(1,4,5)P₃ assay kit [23]. The standard curve was linear from 0.19 to 25 pmol of Ins(1,4,5)P₃.

Measurement of intracellular Ca²⁺ content by digital fluorescence microscopy

The cells (1 × 10⁴ cells/well) were plated on a glass coverslip which adhered to the smooth lower side of a Flexiperm-Disc (Heraeus Biotechnology, Hanau, Germany) for 24–36 h at 37 °C. Before stimulation with gangliosides, these cells were washed twice with fresh L-MEM and loaded with the Ca²⁺ indicator fura-2/AM (2 µM) by incubation for 30 min at 37 °C in 0.5 ml of L-MEM.

The cells were washed twice with fresh L-MEM and exposed to 1–25 µg of gangliosides/ml. Fluorescence images were obtained at alternating excitation wavelengths of 340 and 380 nm through a SIT vidicon camera, and processed using an ARGUS-100 image analyser (Hamamatsu Photonics Corp., Hamamatsu, Japan). The calibration of the fluorescence signal in terms of [Ca²⁺]_i was performed using a digital imaging microscope as described [24,25].

PKC

Cells grown in L-MEM were treated with 5–25 µg of gangliosides/ml and then washed twice with cold Hanks' balanced salt solution and scraped from the culture dishes. The cell suspension was centrifuged for 5 min at 200 g and resuspended in 25 mM-Tris/HCl buffer, pH 7.5, containing 2 mM-EGTA, 5 mM-dithiothreitol (DTT) and 1 mM-phenylmethanesulphonyl fluoride. Preparation of cytosol and membrane fractions was carried out as described [26]. PKC activity was assayed by measuring the incorporation of ³²P from [γ-³²P]ATP into histone H-1 (type III-S) as described previously [27].

Measurement of DNA synthesis

Cells grown in L-MEM were treated with gangliosides at concentrations of 1–50 µg/ml and then labelled for 4 h with 1.0 µCi of [³H]thymidine/ml. After three washings with phosphate-buffered saline (PBS), the cells were lysed with 2 M-NaOH at 37 °C for 20 min and then neutralized with 2 M-HCl. Acid-insoluble material was precipitated by addition of 4 vol. of ice-cold 10 % trichloroacetic acid and collected on GF/A glass fibre filters (Whatman, Ann Arbor, MI, U.S.A.) [10]. After three washings with 10 % trichloroacetic acid and one with ethanol, the filters were dried at room temperature for measurement of radioactivity.

Assay for transglutaminase activity

Cells were treated with G_{Q1b} or its derivatives for the indicated periods of time. After standing overnight, the cells were washed with 2 ml of cold Dulbecco's PBS (pH 7.2) and scraped carefully

with a scraper after adding 1 ml of PBS. This washing step was then repeated, and the cell suspensions were centrifuged at 200 g for 5 min. Washed cells were resuspended in 10 mM-Tris/HCl buffer, pH 7.4, containing 10 mM-DTT, 0.5 mM-EDTA-Na and 1 % Triton X-100. The suspensions were stirred on ice for 30 min, and then subjected to centrifugation at 105000 g for 60 min. The final supernatants were used for the transglutaminase assay. Transglutaminase activity was assayed by measuring the incorporation of [¹⁴C]putrescine into dimethyl-casein as described previously [12,28].

Scoring of cornified envelopes

The scoring of cornified envelope formation was performed according to the procedure of Sun & Green [29,30]. To calculate the percentages of cells containing SDS-insoluble and 2-mercaptoethanol (ME)-resistant cornified envelopes, the cells cultures were washed with PBS and the attached cells were dissociated by treatment with 0.25 % trypsin and 0.1 % EDTA-Na solution at 37 °C for 10 min. The suspension was centrifuged at 1000 g for 10 min and the cells were resuspended in 10 mM-Tris/HCl buffer, pH 7.4, containing 1 % SDS and 1 % ME at a density of (0.8–2.0) × 10⁶ cells/ml. The suspensions were kept at room temperature for 15 min and then SDS- and ME-resistant cornified envelopes were scored on a haemocytometer.

RESULTS

Effects of gangliosides on formation of cornified envelopes

Since the development of cornified envelopes marks the terminal differentiation of keratinocytes, the effects of various gangliosides on mouse keratinocyte differentiation were examined by measuring the cornified envelope formation. This specific structure is formed beneath the plasma membrane and is resistant to detergents and reducing agents. When G_{Q1b} was added to the keratinocytes at a concentration of 15 µg/ml, cornified envelope formation increased approx. 4-fold compared with the control. In contrast, cells treated with other gangliosides, e.g. G_{T1b} and G_{M1}, did not show increased formation of cornified envelopes.

Effects of gangliosides on DNA synthesis

The effect of gangliosides on keratinocyte proliferation was examined by measuring [³H]thymidine incorporation into these cells over 4 h. The addition of G_{Q1b} at various concentrations caused a dose-dependent inhibition of DNA synthesis. Almost complete inhibition of DNA synthesis was observed at a G_{Q1b} concentration of 40 µg/ml (Fig. 1). Other gangliosides, i.e. G_{M1} and G_{D1b}, were virtually ineffective, although G_{T1b} caused a small suppression of DNA synthesis. In order to evaluate the potency of G_{Q1b} to induce differentiation in mouse keratinocytes, we further studied its effects on DNA synthesis by comparing them with those caused by a representative differentiating compound, i.e. 1α,25(OH)₂D₃. When [³H]thymidine incorporation into the cells was examined, both G_{Q1b} and 1α,25(OH)₂D₃ caused marked inhibition, reaching a maximum at 20–66 h after exposure (Fig. 2), indicating that DNA synthesis was decreased. The extent of suppression induced by G_{Q1b} (25 µg/ml) was similar to or rather greater than that in 1α,25(OH)₂D₃-treated cells (50 nM).

Ins(1,4,5)P₃ accumulation induced by G_{Q1b}

Since phosphoinositide turnover has been suggested to be associated with keratinocyte differentiation, the mass content of Ins(1,4,5)P₃ was measured in cells stimulated with various gangliosides, i.e. G_{Q1b}, G_{T1b}, G_{D1b}, and G_{M1}, for different periods

Fig. 1. Dose-dependent suppression of DNA synthesis by G_{Q1b}

The cells were cultured in L-MEM for 24 h with various concentrations of the ganglioside derivatives G_{D1b} (X), G_{M1} (●), G_{T1b} (□) or G_{Q1b} (○). Measurement of DNA synthesis ($[^3H]$ thymidine incorporation) was performed as described in the Experimental section. Values are means of triplicate determinations from two separate experiments.

Fig. 2. Time course of suppression of DNA synthesis by G_{Q1b} or $1\alpha,25(OH)_2D_3$

The cells were cultured in L-MEM for the indicated times in the presence of $25 \mu g$ of G_{Q1b}/ml (X), $50 \text{ nM-}1\alpha,25(OH)_2D_3$ (●) or vehicle (○). The extracellular Ca^{2+} concentration was $25 \mu M$. All other experimental details are described in the Experimental section. Values are means of triplicate determinations from three separate experiments.

(0, 30, 60 and 120 s) of incubation. Only G_{Q1b} elicited prominent production of $Ins(1,4,5)P_3$. The $Ins(1,4,5)P_3$ concentration increased immediately after the addition of G_{Q1b} and the peak was attained at 60 s after stimulation, followed by gradual decrease (Fig. 3). The cellular $Ins(1,4,5)P_3$ content was enhanced about 8-fold above the control (from the unstimulated level of $0.69 \pm 0.16 \text{ pmol}/10^5$ cells to a peak of $5.75 \pm 0.33 \text{ pmol}/10^5$ cells) within 60 s when stimulated with $25 \mu g$ of G_{Q1b}/ml . Such a transient increase in $Ins(1,4,5)P_3$ levels caused us to examine $[Ca^{2+}]_i$ changes.

Measurement of $[Ca^{2+}]_i$

Various lines of evidence have revealed the importance of a $[Ca^{2+}]_i$ increase during keratinocyte differentiation, including

Fig. 3. Time course of G_{Q1b} -induced $Ins(1,4,5)P_3$ formation in mouse keratinocytes

Mouse keratinocytes were stimulated with $25 \mu g$ of G_{Q1b}/ml for indicated times. The mass levels of $Ins(1,4,5)P_3$ were assayed as described in the Experimental section. Values are means of triplicate determinations from three separate experiments.

Fig. 4. G_{Q1b} -induced increases in $[Ca^{2+}]_i$

The keratinocytes were loaded with the Ca^{2+} indicator fura-2/AM ($2 \mu M$) by incubation for 30 min at $37^\circ C$ and were then washed twice with fresh medium. (a) Cells were washed with L-MEM ($25 \mu M-Ca^{2+}$) after loading with fura-2/AM, and exposed to $15 \mu g$ of G_{Q1b}/ml at $37^\circ C$. (b) Cells were washed with L-MEM containing $200 \mu M$ -EGTA after loading with fura-2/AM in L-MEM, and exposed to $15 \mu g$ of G_{Q1b}/ml at $37^\circ C$. The arrows indicate the time of G_{Q1b} addition. The ratios of fluorescence intensity at 340 nm and 380 nm have been converted to Ca^{2+} concentration. All other experimental details are described in the Experimental section.

Table 1. Dose-dependent stimulation of transglutaminase activity by G_{Q1b}

The cells were cultured in L-MEM for 24 h in the presence of various concentrations of G_{Q1b} or 50 nM- $1\alpha,25(OH)_2D_3$, and the cells were washed twice. After solubilization of membrane fractions, transglutaminase activity was assayed by measuring the incorporation of [^{14}C]putrescine into dimethylcasein. Other experimental details are described in the Experimental section. Values are means $\pm$ S.D. of triplicate determinations from three separate experiments.

Treatment	Concentration	Transglutaminase activity (% of control)
Control	—	100
$1\alpha,25(OH)_2D_3$	50 nM	229.8 ± 30.9
G_{Q1b}	1 μ g/ml	138.1 ± 16.7
G_{Q1b}	5 μ g/ml	185.1 ± 10.1
G_{Q1b}	25 μ g/ml	259.5 ± 29.8

Fig. 5. Time course of activation of transglutaminase by G_{Q1b}

The cells were cultured in L-MEM for the indicated times in the presence of 25 μ g of G_{Q1b} /ml. The experimental details are as described in the legend to Table 1.

Table 2. Effects of H-7 and down-regulation of PKC on G_{Q1b} -induced cornified envelope formation

Keratinocytes were preincubated with 30 μ M-H-7, 2 μ M-PdBu or vehicle for 30 h and then incubated with 25 μ g of G_{Q1b} /ml for 4 days. Incubations were terminated and then cornified envelopes were scored as described in the Experimental section. Values are means $\pm$ S.D. of the triplicate determinations from three separate experiments.

Treatment	Cornified envelope formation (% of control)
Control	100
G_{Q1b}	463.9 ± 47.8
H-7 plus G_{Q1b}	193.5 ± 49.9
PdBu plus G_{Q1b}	226.1 ± 23.9

Table 3. Effects of H-7 and down-regulation of PKC on G_{Q1b} -induced activation of transglutaminase

Cells pretreated with 30 μ M-H-7, 2 μ M-PdBu or vehicle for 30 h were then incubated with 25 μ g of G_{Q1b} /ml for 24 h. After solubilization of membrane fractions, transglutaminase activity was assayed as described in the Experimental section. Values are means $\pm$ S.D. of triplicate determinations from three separate experiments.

Treatment	Transglutaminase activity (% of control)
Control	100
G_{Q1b}	312.3 ± 27.4
H-7 plus G_{Q1b}	120.5 ± 30.2
PdBu plus G_{Q1b}	178.1 ± 11.1

that induced by $1\alpha,25(OH)_2D_3$. $[Ca^{2+}]_i$ levels were investigated in keratinocytes loaded with fura-2/AM by single-cell Ca^{2+} imaging analyses. In the presence of 25 μ M extracellular Ca^{2+} , the addition of G_{Q1b} (25 μ g/ml) resulted in a rapid increase in $[Ca^{2+}]_i$, peaking at 60 s after stimulation and then slowly declining. A representative profile is displayed in Fig. 4. The maximal $[Ca^{2+}]_i$ responses were obtained at 15–30 μ g of G_{Q1b} /ml, whereas little rise in $[Ca^{2+}]_i$ was observed with the same concentrations of G_{D1b} (results not shown). At G_{Q1b} concentrations of 15–30 μ g/ml more than 85% of mouse keratinocytes (over 80 cells) exhibited rapid $[Ca^{2+}]_i$ rises. The transient increases in $(Ca^{2+})_i$ were not affected by chelating the extracellular Ca^{2+} with excess EGTA, but the peak $[Ca^{2+}]_i$ level was decreased to a small extent, which was followed by a decline back to the control level within 5 min. This indicates that the $[Ca^{2+}]_i$ rise is principally due to $Ins(1,4,5)P_3$ -mediated Ca^{2+} mobilization from internal stores. Since Ca^{2+} is well known as a crucial regulator of numerous cellular functions, our results also suggest that Ca^{2+} is a primary messenger for the action of G_{Q1b} leading to mouse keratinocyte differentiation.

Effects of gangliosides on transglutaminase activity

Transglutaminase activity is a critical enzymic marker of keratinocyte differentiation. The increase in $[Ca^{2+}]_i$ during differentiation has been shown to be implicated in the activation of this Ca^{2+} -requiring enzyme, which catalyses the cross-linking of membrane proteins to form cornified envelopes. We examined the effects of G_{Q1b} on transglutaminase activity in epidermal keratinocytes which had been incubated with the ganglioside for 24 h. The enzyme activity was increased in a dose-dependent manner (Table 1). The exposure of mouse keratinocytes to G_{Q1b} (25 μ g/ml) for various incubation periods caused a gradual increase in transglutaminase activity, reaching a peak at around 40 h and returning to the basal level between 60 and 72 h (Fig. 5).

Effects of H-7 and chronic exposure to phorbol ester on G_{Q1b} -induced keratinocyte differentiation

The participation of PKC in keratinocyte differentiation has been proposed as a result of studies using phorbol esters [9,31]. PKC isoenzymes were examined by Western blot analysis using monoclonal antibodies specific for each PKC isoform. It was shown that mouse keratinocytes express α but not β or γ PKC. Our previous results demonstrated that $1\alpha,25(OH)_2D_3$ -induced formation of cornified envelopes was inhibited by the treatment with the PKC inhibitor H-7 or by a prolonged incubation with phorbol 12,13-dibutyrate (PdBu) [14]. The PKC activity was undetectable after a 24 h incubation with 2 μ M-PdBu. We here examined the effects of these treatments on cornified envelope

Fig. 6. Time course of translocation of PKC induced by G_{Q1b} and G_{D1b}

The cells were treated with 15 μ g of G_{Q1b} (○, ●) or G_{D1b} (△, ▲) for the indicated times. After preparation of cytosol (○, △) and membrane (●, ▲) fractions, PKC activities were assayed by measuring the incorporation of ³²P into histone H-1. Other experimental details are described in the Experimental section.

formation induced by G_{Q1b}. It was demonstrated that the mouse keratinocytes so treated exhibited marked suppression of G_{Q1b}-induced formation of cornified envelopes (Table 2). However, complete inhibition was not observed under these conditions, whereas 1 α ,25(OH)₂D₃-induced cornification was completely suppressed, as reported previously [14]. Furthermore, under the same conditions, the activation of transglutaminase by G_{Q1b} was markedly decreased (Table 3). These results strongly suggest that G_{Q1b}-induced transglutaminase activation may be linked to PKC.

G_{Q1b}-induced translocation of PKC activity

As membrane association is thought to represent an initial step in PKC activation, we have examined whether G_{Q1b} causes a redistribution of PKC. Fig. 6 shows a rapid translocation of PKC from cytosol to membrane. The cytosolic PKC activity decreased from an unstimulated level of 161 $\pm$ 10 pmol/min per mg of protein to 127 $\pm$ 11 pmol/min per mg of protein within 2–5 min after stimulation by G_{Q1b}, and a concomitant increase in membrane-associated PKC activity was observed (from an unstimulated level of 16.7 $\pm$ 3.4 pmol/min per mg of protein to a peak of 54.2 $\pm$ 9.2 pmol/min per mg of protein). In contrast, another ganglioside, G_{D1b}, which is incapable of eliciting keratinocyte differentiation, did not result in PKC translocation from cytosol to membrane.

DISCUSSION

Mouse epidermis has been a useful model for analysing tissue differentiation, and many studies have been performed with cultured keratinocytes. Although morphological and biochemical information is abundant, the precise mechanism by which the differentiation process is induced has not been fully understood. It is widely known that increasing the extracellular Ca²⁺ concentration and 1 α ,25(OH)₂D₃ both provoke terminal differentiation of cultured keratinocytes [4]. Recent studies have provided

evidence indicating that intracellular Ca²⁺ mobilization coupled with phosphoinositide turnover is of primary importance as a trigger for the onset of the signal transduction for keratinocyte terminal differentiation [4,32].

We have previously demonstrated that when 1 α ,25(OH)₂D₃ was added to primary cultures of keratinocytes isolated from newborn Balb/c mice, a transient increase in the mass of Ins(1,4,5)P₃ occurred concurrently with a considerable elevation of [Ca²⁺]_i [14]. Furthermore, it was postulated from the results of experiments using some vitamin D₃ analogues having no differentiative ability that PKC activation was necessary but not sufficient in itself for induction of terminal differentiation. Exposure of keratinocytes to 1 α ,25(OH)₂D₃ is known to induce phosphoinositide turnover, generating Ins(1,4,5)P₃, but the mechanism(s) of activation of phospholipase C by the steroid hormone, which reacts with receptors in cytoplasm, remains to be disclosed. Thus this is not a typical case of transmembrane signal transduction through the plasma membrane, as observed in many secretory cell types.

Increasing attention has been focused on the roles of gangliosides in cellular regulation, including differentiation [15]. Tsuji *et al.* [21] showed evidence for induction by tetrasialoganglioside G_{Q1b} of differentiation (neurite outgrowth) of human neuroblastoma cell lines. The action of G_{Q1b} was considered to be initiated by interaction with the plasma membrane, but the mode of interaction is not known. Other gangliosides, such as G_{D1a}, G_{D1b}, G_{T1a} and G_{T1b}, were ineffective. In the present study we have examined whether gangliosides can cause differentiation of keratinocytes. Among the compounds tested, only G_{Q1b} was found to be active in the formation of cornified envelopes, a characteristic marker of keratinocyte terminal differentiation. The biochemical events in G_{Q1b}-treated cells were similar to those obtained in keratinocytes exposed to high Ca²⁺ or to 1 α ,25(OH)₂D₃. However, the profiles of the rise in [Ca²⁺]_i showed some differences: the G_{Q1b}-induced [Ca²⁺]_i increase was transient, whereas the high-Ca²⁺-induced [Ca²⁺]_i rise was rather sustained [31–34]. However, the mechanism for this sustained elevation remains unknown.

The enhancement of the [Ca²⁺]_i level was associated with a several-fold increase in Ins(1,4,5)P₃ mass. In the presence of EGTA in the culture medium a small decrease was observed in the peak [Ca²⁺]_i level (Fig. 4), indicating that Ins(1,4,5)P₃-induced intracellular Ca²⁺ mobilization plays a major role in the [Ca²⁺]_i rise elicited by G_{Q1b}. It is well documented that Ca²⁺ is a necessary prerequisite for the differentiation of keratinocytes [31–34]. For example, Ca²⁺ is required for certain features of both the early and the late stages of keratinocyte differentiation, such as desmosome formation and transglutaminase activation. This Ca²⁺-requiring transglutaminase is essential for formation of the cross-linked envelope. Indeed, G_{Q1b} caused a dose- and time-dependent elevation of transglutaminase activity in cultured mouse keratinocytes (Fig. 5 and Table 1). The pleiotropic effects of Ca²⁺ also include PKC activation, which may be associated with keratinocyte differentiation. Yuspa *et al.* [8,32] suggested by using phorbol esters that differentiation is linked to the activation of PKC. We have demonstrated here that, in mouse keratinocytes pretreated with PKC inhibitor H-7 or chronically exposed to phorbol ester (24 h) to down-regulate PKC activity, cornified envelope formation as well as transglutaminase activation induced by G_{Q1b} were markedly decreased. These findings may indicate involvement of PKC in G_{Q1b}-mediated terminal differentiation of epidermal keratinocytes. However, the signal pathway via phosphorylation after PKC activation is not clearly understood. Recently, Chakravarty *et al.* [35] have shown that phorbol ester phosphorylates keratinocyte transglutaminase in the membrane anchorage region, and they suggested that phosphorylation

may modulate interaction of the enzyme with specific substrate proteins, e.g. involucrin. In order to specify the site and mode of action of PKC in epidermal terminal differentiation, further investigations are required.

In conclusion, the results presented here provide evidence that tetrasialoganglioside G_{Q1b} induces terminal differentiation of mouse keratinocytes, and further suggest that the acute increase in $[Ca^{2+}]_i$ elicited by phosphoinositide breakdown is essential for the onset of differentiation.

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