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Inhibition of Protein Prenylation Down-Regulates Signalling by Inflammatory Mediators in Human Keratinocytes

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Several inflammatory mediators have been shown to activate phospholipase C in human keratinocytes via GTP-binding protein-coupled receptors. Since GTP-binding proteins are prenylated proteins, we have examined the role of prenylation in signal transduction in HaCaT keratinocytes. Indirect inhibition of prenylation with the HMG CoA reductase inhibitors fluvastatin or compactin decreased bradykinin-stimulated inositol 1,4,5-triphosphate generation. This effect was abolished by mevalonic acid but not by serum, indicating a requirement for a non-sterol metabolite for signal generation. The BK response was also inhibited by zaragozic acids B and C, known inhibitors of prenyl protein transferases. These results suggest that protein prenylation may be a novel therapeutic target in dermatological conditions where an up-regulation of the inositol lipid pathway has been demonstrated.

One of the most widely studied signal generating pathways is the breakdown of phosphatidylinositol 4,5-bisphosphate. This lipid is hydrolysed through the activation of phospholipase C (PLC) to generate the two second messengers inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) and sn 1,2-diacylglycerol which mediate the release of intracellular calcium and activation of protein kinase C (PKC) respectively [1,2]. Activation of the inositol lipid pathway has been linked to fundamental cellular responses such as proliferation, differentiation and inflammation. The regulation of this pathway in the skin is of considerable interest due to its potential involvement in the pathogenesis of inflammatory and hyperproliferative skin diseases such as psoriasis. Psoriatic epidermis shows abnormalities in several components of this system including PKC, PLC and phosphatidylinositol kinase [3].

Inflammatory mediators such as bradykinin (BK) and platelet-activating factor (PAF) have been shown to activate PLC in human keratinocytes and there is evidence that the receptors for these mediators are coupled to PLC via a GTP-binding protein (G protein) [4,5,6]. G proteins are heterotrimeric proteins composed of a, β and γ subunits [7] of which the γ subunit is modified by a geranylgeranyl isoprenoid chain [8,9]. This modification, termed prenylation, has been shown to be important for the membrane association and activity of G protein subunits.

Nothing is known about the role of prenylated proteins in signalling processes in keratinocytes. We were therefore interested to investigate the importance of prenylation for stimulation of the inositol lipid pathway in the human keratinocyte cell line HaCaT. Prenylation was indirectly inhibited using fluvastatin and compactin, inhibitors of HMG CoA reductase. This enzyme regulates the production of mevalonic acid, a key intermediate in the cholesterol biosynthetic pathway and for isoprenoid synthesis. HMG CoA reductase inhibitors have previously been shown to inhibit some other signal-dependent cellular processes such as mitogen-stimulated cell growth, DNA synthesis and gene expression and mast cell degranulation [8,9]. We show here that inhibition of HMG CoA reductase leads to down-regulation of receptor-mediated $Ins(1,4,5)P_3$ generation stimulated by selected inflammatory mediators. We further demonstrate that direct inhibitors of protein

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Abbreviations: PLC, phospholipase C; $Ins(1,4,5)P_3$, inositol 1,4,5-trisphosphate; PKC, protein kinase C; BK, bradykinin; PAF, platelet-activating factor; G protein, GTP-binding protein.

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prenylation have a similar effect suggesting that protein prenylation is essential for coupling of receptor activation to second messenger generation.

MATERIALS AND METHODS

Materials. Compactin was from Fluka. Zaragozic acids B and C were a gift from Dr. Traber, Sandoz Pharma, Basel. NB-598 and fluvastatin were synthesized by the Sandoz company.

Cell culture. The HaCaT cell line which was supplied by Prof. N. Fusenig (Heidelberg) is a human keratinocyte cell line [10]. HaCaT cells were routinely maintained in Keratinocyte Basal Medium (KBM from Clonetics) supplemented with bovine pituitary extract (2ml/500ml medium), EGF (10μg/ml, 0.5ml/500ml medium), insulin (5mg/ml, 0.5ml/500ml medium), hydrocortisone (0.5mg/ml, 0.5ml/500ml medium) and GA-1000 (Gentamicin 50mg/ml and Amphotericin-B 50μg/ml, 0.5ml/500ml medium); the medium is then referred to as Keratinocyte Growth Medium (KGM). The cells were grown in a humidified atmosphere of 5% CO_2 at 37°C and the medium was changed every two days.

Measurement of Ins(1,4,5)P_3. For Ins(1,4,5)P_3 measurements keratinocytes were grown to 70–80% confluency in 6-well plates after which the medium was changed to KBM basal medium and the cells incubated for a further 36–48 hours. The medium was then aspirated and the cells washed once with 2ml PBS and once with 2ml KBM. The cells were stimulated at 37°C in 1ml KBM to which was added 10μl of agonist. After the required time the reaction was stopped with 0.2ml of ice-cold 20% (v/v) perchloric acid and the plates left on ice for 20 min. The cells were then scraped and the contents of each well transferred to plastic tubes. These were centrifuged at 2,000g for 15 min at 4°C. The supernatant was removed and the pH adjusted to 7.5 with 60mM Hepes/1.5M KOH. The samples were then centrifuged again at 2,000g for 15 min at 4°C and the supernatant removed. Aliquots of 100μl were transferred to eppendorf tubes and Ins(1,4,5)P_3 levels measured using an Ins(1,4,5)P_3 assay system from Amersham. The assay is based on the competition between unlabelled Ins(1,4,5)P_3 and a fixed quantity of tritium-labelled Ins(1,4,5)P_3 for a limited number of binding sites on a bovine adrenal binding preparation.

Radiolabelling of cellular proteins with [^{3}H] mevalonic acid. Cells were grown to 90% confluency in KGM in 24-well plates and then incubated for 16 hours with 10μM fluvastatin in KBM. The medium was aspirated and the cells incubated with fresh KBM containing 10μM fluvastatin and 100μCi/ml of [^{3}H] mevalonolactone (10–30Ci/mmol, NEN) for 6–8 hours. The medium was then removed and the cells washed 3× with cold PBS. 50μl of lysis buffer (50mm Tris, pH 7.6; 1% (v/v) Triton X-100; 120mM NaCl; 5mM MgCl2; 5mM KCl; 10μl/ml leupeptin; 1mM PMSF; 1μg/ml aprotinin; 10μg/ml pepstatin A) was added and the plates left on ice for 20 min. The cells were scraped and the samples transferred to eppendorf tubes. Following centrifugation at 12,000g for 15min at 4°C, 15μl of the supernatant was removed and mixed with 30μl of sample buffer (0.0625M Tris, pH 6.8; 2% (w/v) SDS; 5% (v/v) β-mercaptoethanol; 0.025% (w/v) bromophenol blue). The samples were heated for 5 min at 95°C and 20μl samples were then fractionated on an ExcelGel 8–18 gradient gel (Pharmacia). Gels were fixed in 40% (v/v) methanol/10% (v/v) acetic acid followed by a 15–30 min incubation in Amplify fluorographic reagent (Amersham). Gels were exposed to Hyperfilm-MP (Amersham) for 5 days and the films developed using a Curix 60 automatic processor.

RESULTS AND DISCUSSION

Since G-proteins have been shown to belong to the wide range of proteins which are prenylated we were interested to investigate the importance of prenylation for signal transduction in keratinocytes. In HaCaT cells BK at a concentration of 1μM stimulated a rapid rise in Ins(1,4,5)P_3 generation which was maximal after 15 sec (Fig. 1). We investigated the effect of fluvastatin on inositol phosphate generation by pretreating cells for 1 hour or 16 hours with 10μM of the compound prior to stimulation with BK. Fluvastatin inhibits HMG CoA reductase [11] which catalyses the reduction of HMG CoA to mevalonic acid in the cholesterol biosynthetic pathway and inhibiting this enzyme therefore blocks the supply of prenyl units. In cells pretreated for just 1 hour the stimulation of Ins(1,4,5)P_3 generation was the same as in untreated cells (data not shown). However, after 16 hour pretreatment the response to BK was reduced by approximately 50% compared to control cells which had been pretreated with DMSO (Fig. 1). We also investigated the effect of pretreatment with compactin, an HMG CoA reductase inhibitor with a different chemical structure to fluvastatin. Following pretreatment with 10μM compactin, the Ins(1,4,5)P_3 response to BK was also reduced (DMSO pretreatment: 51.0 ± 8.1 pmol/sample; Compactin pretreatment: 33.0 ± 6.2 pmol/sample). This confirms that the effect is not specific to fluvastatin but rather is as a result of inhibition of HMG CoA reductase by diverse compounds. Deanin et al. [12] showed a similar inhibition of Ins(1,4,5)P_3 generation in response to antigen-stimulation in RBL-2H3 (a rat basophilic leukemia cell line) with lovastatin, another HMG CoA reductase inhibitor.

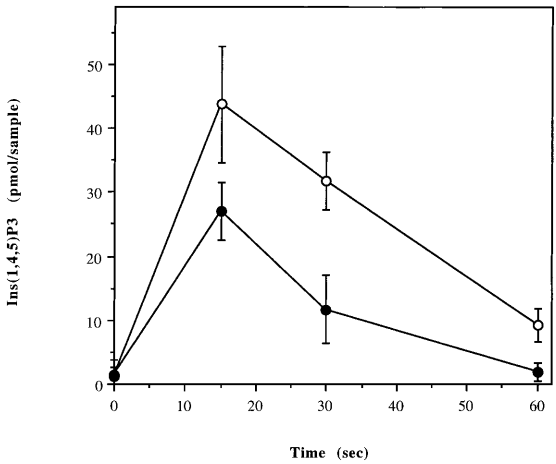

FIG. 1. Effect of fluvastatin pretreatment on BK-stimulated Ins(1,4,5)P_3 generation in HaCaT cells. HaCaT cells were pretreated with 10 μM fluvastatin (●) or DMSO (○) for 16 hours. The cells were then stimulated with 1 μM BK for the times shown and the amount of Ins(1,4,5)P_3 produced measured as described. Each value is the mean ± S.D. of triplicate determinations. The data are representative of three different experiments.

In order to confirm that the inhibition was indeed due to the inhibition of HMG CoA reductase, the effect of fluvastatin pretreatment in the presence of exogenously added mevalonic acid was investigated. In the presence of 10mM mevalonic acid the inhibitory effect of fluvastatin on BK-stimulated Ins(1,4,5)P_3 generation in HaCaT cells was abolished (Fig. 2) suggesting that a metabolite of mevalonic acid is required for signal generation.

Since inhibition of HMG CoA reductase results in an inhibition of cholesterol biosynthesis, the reduction in stimulated Ins(1,4,5)P_3 levels could be a consequence of a lack of cholesterol. Experiments were therefore carried out in the presence and absence of foetal calf serum as a source of exogenous cholesterol. However, even in the presence of serum, pretreatment with fluvastatin resulted in a decrease in BK-stimulated Ins(1,4,5)P_3 production (Table 1). Similarly, pretreatment

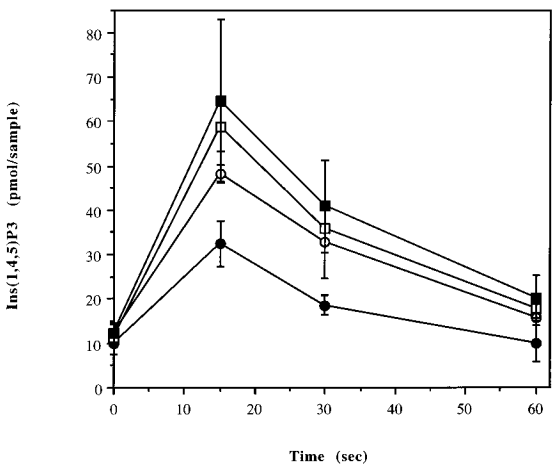

FIG. 2. Mevalonic acid abolished the inhibitory effect of fluvastatin on BK-stimulated Ins(1,4,5)P_3 generation. HaCaT cells were pretreated for 16 hours with 10μM fluvastatin (closed symbols) or DMSO (open symbols) in the presence (■,□) or absence (●,○) of 10mM mevalonic acid. The cells were then stimulated with 1μM BK for the times shown and the Ins(1,4,5)P_3 levels measured as described. Each value is the mean ± S.D. of triplicate determinations. The data are representative of three different experiments.

TABLE 1
Effect of Fluvastatin and NB-598 on BK-Stimulated Ins(1,4,5)P_3 Generation in the Presence and Absence of Foetal Calf Serum

	Ins(1,4,5)P_3			
	Without Serum		10% Serum	
	Control	BK	Control	BK
DMSO pretreatment	6.6 ± 1.9	37.5 ± 3.0	4.8 ± 0.4	21.9 ± 3.89
Fluvastatin pretreatment	9.1 ± 2.6	24.3 ± 3.4	5.1 ± 2.04	12.1 ± 1.6
	Control	BK	Control	BK
DMSO pretreatment	5.5 ± 3.9	79.5 ± 20.6	5.7 ± 4.3	62.7 ± 20.8
NB-598 pretreatment	6.3 ± 2.4	95.1 ± 9.4	11.3 ± 2.7	72.6 ± 12.7

HaCaT cells were pretreated with 10μM fluvastatin, 10μM NB-596 or DMSO in the presence or absence of 10% foetal calf serum for 16 hours. The cells were then stimulated with 1 μM BK for 15sec and Ins(1,4,5)P_3 levels measured as described. Each value is the mean ± S.D. of triplicate determinations. The data are representative of three different experiments.

of cells for 16 h with NB-598, a potent and specific squalene epoxidase inhibitor [13] which therefore inhibits cholesterol biosynthesis downstream of mevalonic acid synthesis, had no effect on the BK response either in the presence or absence of serum (Table 1). These results indicate that the inhibition of second messenger generation is related to a lack of a non-sterol intermediate in the cholesterol biosynthetic pathway and that this intermediate is a product of mevalonic acid generated prior to squalene.

We also investigated the effect of fluvastatin on other agonists which stimulate inositol lipid turnover in HaCaT cells. Platelet-activating factor (PAF, 3μM), ATP (100μM) and thrombin (1unit/ml) all stimulated Ins(1,4,5)P_3 production (Fig. 3), but only the response to PAF was inhibited by pretreatment with fluvastatin. The compound had no effect on the response to either ATP or thrombin. This may indicate that these mediators couple to PLC via different pathways which do not involve modified proteins demonstrating that fluvastatin does not non-specifically inhibit all pathways leading to Ins(1,4,5)P_3 generation but rather acts in a specific manner on certain receptor systems.

The experiments with fluvastatin suggested that there was a requirement for a non-sterol me-

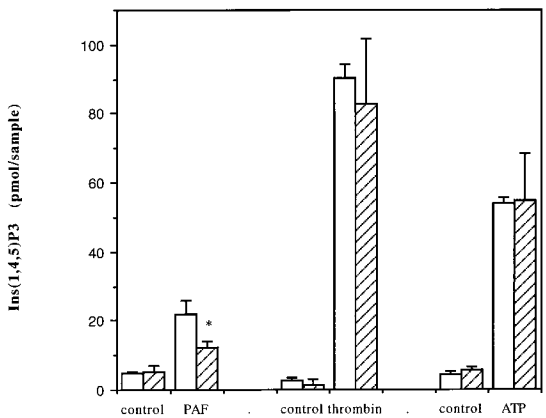

FIG. 3. Effect of fluvastatin pretreatment on Ins(1,4,5)P_3 generation in response to PAF, thrombin and ATP. HaCaT cells were pretreated with 10μM fluvastatin (hatched bars) or DMSO (open bars) for 16 hours. The cells were then stimulated with 3μM PAF, 100μM ATP or 1unit/ml thrombin for 15sec and Ins(1,4,5)P_3 levels measured as described. Each value is the mean ± S.D. of triplicate determinations. The data are representative of three different experiments. *$p < 0.01$

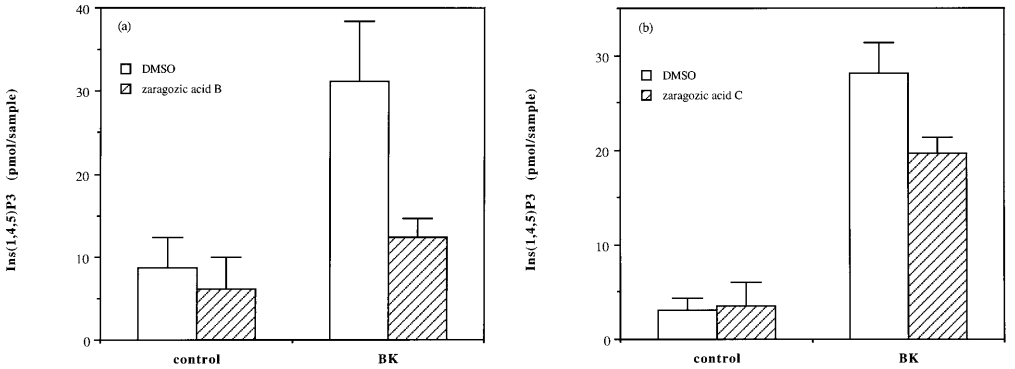

FIG. 4. Zaragozic acids B and C inhibited BK-stimulated Ins(1,4,5)P_3 generation. HaCaT cells were pretreated for 16 hours with (a) 10μM zaragozic acid B or DMSO or (b) 10μM zaragozic acid C or DMSO. The cells were then stimulated for 15 sec with 1μM BK and Ins(1,4,5)P_3 levels measured as described. Each value is the mean ± S.D. of triplicate determinations. The data are representative of three different experiments.

tabolite of mevalonic acid for signal generation and the most likely interpretation of these results is that an isoprenylated protein is involved in signal transmission from the BK receptor. The effect of the prenyl transferase inhibitors, zaragozic acid B and C [14], on BK-stimulated Ins(1,4,5)P_3 formation was therefore investigated. When cells were pretreated with these compounds for 16 hours prior to stimulation there was a 30–60% decrease in Ins(1,4,5)P_3 production (Fig. 4a + 4b). This provides strong support for the proposal that a prenylated protein is involved in signal transmission.

A frequently used method for detecting prenylated proteins in cells is by labelling with [^{3}H] mevalonic acid which can be incorporated into farnesyl and geranylgeranyl isoprenoids, substrates for prenyl protein transferase enzymes, and hence into proteins. We investigated the incorporation of radiolabel from [^{3}H] mevalonolactone into cellular proteins by first pretreating cells with 10 μM fluvastatin for 16 hours to inhibit endogenous mevalonic acid synthesis and subsequently incubating with exogenously added [^{3}H] mevalonolactone. Several protein bands were observed which were in the same molecular weight range as reported in the literature for known prenylated proteins (Fig. 5). The 20–30 kD proteins which were most strongly labelled probably include the small GTP-binding family of proteins, some of which are farnesylated and others geranylgeranylated. The 46, 60 and 80 kD species are probably farnesylated proteins including the nuclear lamins while the 8–10 kD species corresponds in size to the γ subunit of the heterotrimeric GTP-binding proteins [8,9].

The results of our studies indicate that a non-sterol metabolite of mevalonic acid, most likely a

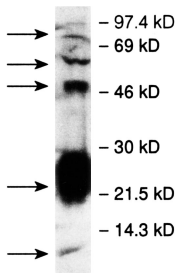

FIG. 5. Radiolabelling of HaCaT cellular proteins with [^{3}H] mevalonic acid. HaCaT cells were pretreated for 16 hours with 10μM fluvastatin. The cells were then incubated for a further 6–8 hours with 100μCi/ml of [^{3}H] mevalonolactone in the presence of 10μM fluvastatin. Labelled proteins were analysed by SDS PAGE followed by autoradiography. The data are representative of three different experiments.

prenylated protein, is required for coupling of the BK receptor to PLC. The most likely candidate is the γ subunit of a G protein and we have shown that HaCaT cells contain an 8-10kD prenylated protein which is of the correct molecular weight to be a γ subunit. Prenylation of the γ subunit appears to be important for both interaction with receptors and effectors. A possible interpretation of our results is therefore that fluvastatin inhibits the prenylation of the γ subunit of a G protein involved in coupling the BK receptor to PLC. This could then inhibit the interaction of the G protein with the receptor or PLC or both, thus inhibiting the production of Ins(1,4,5)P_3. We have recently reported that HaCaT cells contain prenyl transferase enzymes [15]. This observation together with the results presented here indicating a role for a prenylated protein in PLC activation suggest that prenylation may represent a novel target for therapeutic intervention in dermatological inflammation and hyperproliferative disorders where abnormalities in the PLC signal transduction system are implicated.

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