Differential induction of phosphatidylcholine hydrolysis, diacylglycerol formation and protein kinase C activation by epidermal growth factor and transforming growth factor- α in normal human skin fibroblasts and keratinocytes

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We have investigated coupling between the epidermal growth factor (EGF) receptor and the phospholipase C (PLC)/protein kinase C (PKC) signal-transduction system in normal skin fibroblasts and keratinocytes, for which EGF and transforming growth factor α (TGF- α) are mitogenic. EGF and TGF- α induced a rapid increase in tyrosine phosphorylation of the EGF receptor, in both fibroblasts and keratinocytes, but failed to induce tyrosine phosphorylation of PLC- γ 1 or detectable phosphoinositide hydrolysis, as measured by two sensitive assays. In fibroblasts, EGF induced phosphatidylcholine (PC) hydrolysis, resulting in increased diacylglycerol (DAG). In contrast, in keratinocytes, there was no detectable PC hydrolysis or elevation of DAG in response to EGF or TGF- α . EGF and TGF- α activated PKC in fibroblasts, as evidenced by increased phosphorylation of a specific cellular PKC substrate (myristoylated alanine-rich C-

kinase substrate, 'MARCKS'). In keratinocytes, TGF- α and EGF induced only a modest increase in MARCKS protein phosphorylation. This apparent modest activation of PKC, in the absence of detectable DAG formation, may have been mediated by arachidonic acid, which was released from keratinocytes in response to TGF- α , and has been shown to stimulate PKC activity *in vitro*. These data demonstrate that (1) in dermal fibroblasts and keratinocytes, which express normal levels of EGF receptors, EGF receptor activation is not coupled to tyrosine phosphorylation of PLC- γ 1 or PtdIns hydrolysis, suggesting that these events are not required for the mitogenic activity of EGF or TGF- α in these cells, (2) coupling of EGF receptor to PC hydrolysis is cell-type specific, and (3) in skin fibroblasts, DAG, formed through EGF-induced PC hydrolysis, is capable of activating PKC.

INTRODUCTION

Epidermal growth factor (EGF) and transforming growth factor- α (TGF- α) are structurally related polypeptide growth factors (Massague, 1983). EGF and TGF- α exhibit very similar affinity constants for the EGF receptor (Massague, 1983; Ebner and Derynck, 1991) and, in general, exert similar biological effects. TGF- α , however, appears to be more potent than EGF in some assays (Myrdal et al., 1986; Schreiber et al., 1986; Barrandon and Green, 1987), which may be due to differences in processing of EGF-receptor and TGF- α -receptor complexes (Ebner and Derynck, 1991).

There are now a number of lines of evidence to suggest that TGF- α plays an important role in autocrine and paracrine growth regulation in human skin. EGF and TGF- α are mitogenic for human keratinocytes and dermal fibroblasts (Harper and Savage, 1980; Barrandon and Green, 1987; Pittelkow et al., 1989). TGF- α mRNA and protein are synthesized in cultured human keratinocytes (Coffey et al., 1987; Cook et al., 1991) and in normal epidermis (Coffey et al., 1987; Elder et al., 1989), and are markedly over-expressed in hyperproliferative psoriatic epidermis (Gottlieb et al., 1988; Elder et al., 1989). Furthermore, epidermal hyperplasia and increased papilloma formation were

observed after over-expression of TGF- α in the epidermis of transgenic mice (Vassar and Fuchs, 1991).

The EGF receptor possesses ligand-dependent tyrosine kinase activity that is required for the biological effects of EGF and TGF- α , including cell growth (Chen et al., 1987). The binding of EGF or TGF- α to the EGF receptor results in dimerization of the receptor and activation of its intrinsic tyrosine kinase activity, leading to autophosphorylation (reviewed in Carpenter and Cohen, 1990). Autophosphorylation of the EGF receptor promotes binding and phosphorylation of phospholipase C- γ 1 (PLC- γ 1) (Vega et al., 1992). This results in stimulation of PLC- γ 1 activity, which requires both tyrosine phosphorylation and binding to the EGF receptor (Vega et al., 1992). Activated PLC- γ 1 hydrolyses PtdIns(4,5) P_2 to yield at least three important second messengers including Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 , which mobilize intracellular Ca^{2+} , and 1,2-diacylglycerol (1,2-DAG), the physiological activator of protein kinase C (PKC) (Nishizuka, 1986).

Activation of PLC- γ 1 by EGF and/or TGF- α has been most extensively studied in cells that over-express EGF receptors, i.e. transformed cells (e.g. A431 cells) or cell lines transfected with EGF receptors. Stimulation of PLC- γ 1 and PKC by EGF and/or TGF- α is, however, not well defined in non-transformed

Abbreviations used: DAG, diacylglycerol; EGF, epidermal growth factor; MARCKS, Myristoylated Alanine-Rich C-Kinase Substrate; PC, phosphatidylcholine; PKC, protein kinase C; PLC- γ 1, phospholipase C- γ 1; PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate; TGF- α , transforming growth factor- α .

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cells that express normal numbers of EGF receptors. EGF-induced mitogenesis in the absence of detectable inositol phospholipid hydrolysis has been reported in Balb/c/3T3 fibroblasts (Besterman et al., 1986), and Chinese hamster embryo fibroblasts (IIC9 cells), (Wright et al., 1988). On the other hand, stimulation of PtdIns hydrolysis by EGF has been reported in Balb/MK cells (Moscat et al., 1988), pig thyroid cells (Takasu et al., 1988) and rat hepatocytes (Tsao et al., 1986; Johnson and Garrison, 1987; Hepler et al., 1988). The reason for this difference among different cell types has not been fully explained, although differences in cell clones, experimental conditions, including the presence or absence of serum, and cell state may play some role (reviewed by Whitman and Cantley, 1989).

Agonist-stimulated accumulation of 1,2-DAG may occur through pathways other than PLC-induced PtdIns(4,5) P_2 hydrolysis. Phosphatidylcholine (PC) and phosphatidylethanolamine may also be hydrolysed, by distinct PLCs, to yield 1,2-DAG directly (reviewed by Exton, 1990; Dennis et al., 1991). Alternatively 1,2-DAG may be generated by the sequential action of phospholipase D (PLD) and phosphatidate phosphohydrolase (Bocckino et al., 1987; van Blitterswijk et al., 1991). Indeed, PC hydrolysis through PLC and/or PLD activity may be the major source of agonist-induced 1,2-DAG formation in certain cell types. Moreover, recent evidence suggests that PC hydrolysis may be important in the control of cell growth (Wright et al., 1988; Larrodera et al., 1990). Synthesis *de novo* may also contribute to elevated 1,2-DAG in agonist-treated cells (Farese et al., 1987).

We and others have previously reported EGF-stimulated DAG accumulation through hydrolysis of PC (Wright et al., 1988; Pessin et al., 1990; Fisher et al., 1991), but it remains unclear whether DAG derived from PC can activate PKC. In the present study, we have extended our previous observations in human dermal fibroblasts, to address this issue directly. In addition, we have examined the relationship between EGF receptor activation and the PLC/PKC signal-transduction system in human keratinocytes, a second major cell type in skin, for which EGF and TGF- α are mitogenic.

EXPERIMENTAL

Materials

myo-[3 H]inositol, [3 H]choline, [3 H]myristic acid, [3 H]arachidonic acid, [3 H]oleic acid, [3 H]PC, [14 C]PC, [125 I]-EGF, [32 P] P_i and [γ - 32 P]ATP were purchased from DuPont NEN. The Ins(1,4,5) P_3 -binding assay kit was from Amersham. 1,2-Dioleoylglycerol, cardiolipin, PC, phosphatidylethanol, platelet-activating factor and phosphatidic acid were obtained from Avanti Polar Lipids (Birmingham, AL, U.S.A.). Arachidonic acid and oleic acid were from Serdary Research Laboratories (Port Huron, MI, U.S.A.). *Escherichia coli* DAG kinase was purchased from Lipidex (Westfield, NJ, U.S.A.). Ion-exchange resin (AG1-X8, formate form) was from Bio-Rad Laboratories (Richmond, CA, U.S.A.). [Lysine]-bradykinin, thrombin and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). TGF- α , anti-phosphotyrosine monoclonal antibody and anti-PLC- γ 1 polyclonal and mixed monoclonal antibodies were from Upstate Biotechnology (Lake Placid, NY, U.S.A.). EGF (receptor grade) was from Collaborative Research (Bedford, MA, U.S.A.). Pansorbin was from Calbiochem. Polyclonal rabbit antiserum to myristoylated alanine-rich C-kinase substrate (MARCKS) (Lobaugh and Blackshear, 1990) was generously given by Dr. P. J. Blackshear. Bryostatins were generously given by Dr. G. R. Pettit. All other chemicals were of at least reagent grade.

Cell culture

Dermal fibroblasts and keratinocytes were cultured from keratome biopsies of adult human skin as previously described (Talwar et al., 1989; Fisher et al., 1991). Cells were used for experiments between passages 3 and 6. Except where specified, cells were placed in basal medium (defined as serum-free McCoy's Modified 5A medium for fibroblasts and growth-factor-free MCDB 153 for keratinocytes), at 50–60% confluency, for 48 h before agonist treatment. A431 cells were obtained from American Type Culture Collection (Rockville, MD, U.S.A.), and cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal-bovine serum.

Metabolic labelling of cells

Fibroblasts and keratinocytes were cultured to 50–60% confluency in 35 mm or 60 mm dishes. The cells were washed with solution A (30 mM Hepes, pH 7.3, 10 mM glucose, 3.2 mM KCl, 130 mM NaCl, 1 mM Na $_2$ HPO $_4$ ·7H $_2$ O) and labelled with *myo*[3 H]inositol (2 or 20 μ Ci/ml) in inositol-free basal medium (serum-free DMEM for fibroblasts and growth-factor-free MCDB 153 for keratinocytes) or [3 H]choline (5 μ Ci/ml) in choline-free basal medium for 48 h. For myristic acid labelling, cells were placed in basal medium for 32 h and labelled for 16 h with [3 H]myristic acid (5 μ Ci/ml) in basal medium containing 100 μ g/ml BSA. For labelling with [32 P] P_i , fibroblasts and keratinocytes were placed in basal medium for 48 h, washed with low-phosphate (100 μ M) basal medium, and labelled with [32 P] P_i (100–300 μ Ci/ml) in low-phosphate basal medium containing 100 μ g/ml BSA for 3 h at 37 °C. To measure release of oleic acid and arachidonic acid, 60 mm dishes of keratinocytes were grown in medium containing 1 μ M unlabelled oleic acid or 1 μ M unlabelled arachidonic acid, respectively, for 24 h. Cells were then placed in basal medium containing 0.1% BSA and 1 μ M of the unlabelled fatty acid for 32 h, washed with solution A and then labelled with [3 H]oleic acid or [3 H]arachidonic acid (1 μ Ci/ml) in basal medium, for 16 h.

Measurement of inositol phosphates

[3 H]inositol phosphates were analysed by Dowex ion-exchange chromatography as previously described (Downes et al., 1982). Ins(1,4,5) P_3 levels were determined by a binding assay as described by Fu et al. (1988).

125 I-EGF binding

This was carried out essentially as previously described (Nickoloff and Mitra, 1989). Non-specific binding was determined as cell-associated radioactivity in the presence of 500-fold excess of unlabelled EGF as previously described (Zachary et al., 1986). Parallel dishes of cells were trypsin-treated, and cell numbers determined with a haemocytometer. The dissociation constant (K_d) and receptor number were estimated by Scatchard (1949) analysis.

Immunoprecipitation of PLC- γ 1

Cells were scraped into a buffer as described by Decker et al. (1990). Half of the lysate was preabsorbed with Pansorbin and then immunoprecipitated with anti-PLC- γ 1 rabbit polyclonal antiserum (10 μ l/ml of lysate) or mixed monoclonal antibodies (1.0 μ l/ml of lysate) as described previously (Decker et al., 1990; Meisenhelder et al., 1989).

Immunoblot analysis

Paired cell lysates and PLC- γ 1 immunoprecipitates were separated on 8%-polyacrylamide gels and proteins were transferred to nitrocellulose membranes. Blots were blocked in Ca^{2+} / Mg^{2+} -free PBS containing 0.05% Tween 20 and 5% powdered milk and then incubated with either anti-phosphotyrosine monoclonal antibody diluted 1:1000 or anti-PLC- γ 1 mixed monoclonal antibodies diluted 1:500. After washing, proteins were detected by using the enhanced chemiluminescence Western-blotting detection system (Amersham).

Measurement of choline and phosphocholine

[^3H]Choline-labelled cells were incubated with the specified agonists, and the reactions were terminated as described by Fisher et al. (1991). Dried samples of the aspirated medium and aqueous cellular layer were redissolved in 50% methanol and analysed by t.l.c. using the solvent methanol/0.5% NaCl/30% NH_3 in water (50:50:1, by vol.) as described by Yavin (1976).

Analysis of [^3H]myristic acid metabolites

To measure the conversion of PC into phosphatidylethanol, [^3H]myristic acid-labelled keratinocytes were washed with Ca^{2+} / Mg^{2+} -free PBS to remove unincorporated label and placed, at timed intervals, in fresh basal medium containing 0.5% ethanol for 15 min. Cells were treated with agonists as specified in the text, scraped into 1 ml of ice-cold methanol, and cellular lipids were extracted with chloroform (Preiss et al., 1987). [^3H]Phosphatidylethanol and neutral lipids were analysed by t.l.c. as described by Billah et al. (1989).

Measurement *in vitro* of membrane-associated and cytosolic PLD activity

After preparation of soluble and particulate fraction, membrane-associated and soluble PLD activities were determined as described by Martin (1988) and Wang et al. (1991) respectively. ^{14}C -labelled products were separated by t.l.c. (Wang et al., 1991), detected and quantified by using a PhosphorImager (Molecular Dynamics).

Measurement *in vitro* of PC-specific PLC activity

Cultures of fibroblasts and keratinocytes, in 100 mm dishes, were washed twice in ice-cold Ca^{2+} / Mg^{2+} -free PBS, scraped into buffer A, homogenized, and centrifuged at 100 000 g for 1 h at 4 °C. The pellet was resuspended in buffer A by 10 passages through a 23-gauge needle. Samples of the supernatant and resuspended pellet were assayed for PC-specific PLC activity as described by Wolf and Gross (1985). T.l.c. analysis was performed as described above for the soluble PLD assay.

Measurement of DAG mass

DAG content of dermal fibroblasts and keratinocytes was quantified by its conversion into [^{32}P]phosphatidic acid by *E. coli* DAG kinase, by the procedure of Preiss et al. (1987) with slight modifications (Talwar et al., 1990; Thompson et al., 1990). [^{32}P]Phosphatidic acid formed was quantified with a PhosphorImager.

Measurement of MARCKS protein phosphorylation

[^{32}P]P $_i$ was added to cell cultures for 3 h at timed intervals to ensure equal labelling times and minimize variation in specific radioactivity of cellular ATP. Cells were then incubated with agonists as specified in the text. MARCKS protein was immunoprecipitated as described by Lobaugh and Blackshear (1990). Alternatively, MARCKS protein phosphorylation was assessed in cell lysates as described by Issandou and Rozengurt (1990). Autoradiograms of dried gels were scanned with a laser densitometer. Dried gels were also analysed by using a PhosphorImager; the incorporation of [^{32}P]P $_i$ into MARCKS was quantified and normalized to the total radioactivity in the lane (to allow for variation in labelling and loading). Results were expressed as percentage of control or percentage of PMA response.

Measurement of arachidonic acid release

After labelling of keratinocytes with [^3H]oleic acid or [^3H]arachidonic acid, medium was aspirated and the cells were washed with solution A containing 0.1% BSA to remove unincorporated radioactivity. Cells were placed in 2 ml of basal medium containing 0.1% BSA, at timed intervals, equilibrated for 15 min and then treated with the specified agonists. At the indicated times 0.5 ml of medium was removed, the remainder of the medium was aspirated, and the cells were scraped into ice-cold methanol. Cellular lipids were extracted with chloroform (Preiss et al., 1987), and counted for radioactivity. The sample of medium removed was acidified with HCl and extracted with 1.5 ml of ethyl acetate. The ethyl acetate extracts were dried in scintillation vials, counted for radioactivity and normalized to incorporation of radioactivity into total cellular lipids.

Statistical methods

For measurements of MARCKS protein phosphorylation, comparisons among treatment groups were performed by the analysis of variance method (ANOVA) and the least-significant-difference test. Summary statistics are reported as means $\pm$ S.E.M.

RESULTS

Inositol phosphate formation in response to TGF- α and EGF

Growth-factor-deprived human adult skin fibroblasts and keratinocytes were labelled with *myo*-[^3H]inositol (2 $\mu\text{Ci/ml}$) and treated with EGF and TGF- α in the presence of LiCl for varying periods of time. The concentrations of EGF and TGF- α used were saturating with respect to EGF receptor binding (see below). No detectable increase in [^3H]inositol phosphates was observed (results not shown). To increase sensitivity, fibroblasts and keratinocytes were labelled with 10 times more of *myo*-[^3H]inositol (20 $\mu\text{Ci/ml}$). This resulted in a 10-fold greater incorporation of label into PtdIns. We were, however, still unable to detect any increased formation of [^3H]inositol phosphates in either fibroblasts or keratinocytes in response to EGF and/or TGF- α (Figure 1). PtdIns hydrolysis was, however, readily detectable in response to thrombin and Lys-bradykinin in fibroblasts and keratinocytes, respectively (Figure 1), as previously reported (Wright et al., 1988; Talwar et al., 1989). Since keratinocytes require *myo*-inositol for growth and differentiation, and confluency is associated with decreased EGF receptor expression (Staiano-Coico et al., 1990), we examined the effect of supplementing basal keratinocyte medium with 1 μM *myo*-inositol during labelling, and varying the confluency at which cells were labelled, on EGF- and/or TGF- α -induced PtdIns hydrolysis. No detectable increases in [^3H]inositol phosphates

Figure 1 Time course of EGF- and TGF- α -stimulated inositol phosphate formation in cultured human adult dermal fibroblasts and keratinocytes

Sub-confluent cultures of fibroblasts (a) and keratinocytes (b) labelled with *myo*-[3 H]inositol (20 μ Ci/ml) were treated with thrombin (5 units/ml), EGF (8.2 nM), Lys-bradykinin (1 μ M), and TGF- α (50 nM), as indicated, and [3 H]inositol phosphates were determined, as described in the Experimental section. Data points represent mean values of three experiments (a) or four experiments (b).

were observed in response to either ligand under any condition examined (results not shown).

To increase further the sensitivity and specificity of detection of PtdIns hydrolysis, we measured Ins(1,4,5) P_3 by a sensitive binding assay. In fibroblasts, the resting level of Ins(1,4,5) P_3 was 5.4 ± 1.3 pmol/ 10^6 cells. No significant change was observed in response to EGF, whereas thrombin induced a 7-fold rise in Ins(1,4,5) P_3 , which reached a peak at 20 s (Figure 2a). In keratinocytes, Lys-bradykinin induced a 10-fold rise in Ins(1,4,5) P_3 after 30 s incubation. However, as shown in Figure 2(b), incubation of keratinocytes with TGF- α for between 0.5 and 30 min produced no significant rise in Ins(1,4,5) P_3 .

EGF receptor expression and activation in fibroblasts and keratinocytes

It is well established that growing adult dermal fibroblasts and keratinocytes express functional EGF receptors (Schaudies et al., 1985; Nickoloff and Mitra, 1989; Khandke et al., 1991). One possible explanation for the observed lack of EGF/TGF- α -induced PtdIns turnover is that EGF receptors were down-regulated during the 48 h of culture in growth-factor-free basal medium, before exposure to EGF and/or TGF- α . We therefore determined EGF receptor number and affinity in fibroblasts and keratinocytes under culture conditions similar to those we had employed to measure EGF/TGF- α -induced PtdIns hydrolysis, i.e. after culture for 48 h in basal growth-factor-free medium. Figure 3 displays the concentration-dependence of EGF binding

in cultured fibroblasts (a) and keratinocytes (b). In fibroblasts, Scatchard analysis yielded a linear plot, with a calculated K_d of 1.81 nM and 66 800 EGF receptors per cell (Figure 3a, insert). In keratinocytes, cultured for 48 h in basal medium, a curvilinear Scatchard plot was obtained (Figure 3b, insert), indicating both high- ($K_d = 0.738$ nM, 51 600 receptors per cell) and low ($K_d = 5.82$ nM, 175 500 receptors per cell) affinity classes of EGF receptors. These values are consistent with other measurements of EGF receptor number and affinity in fibroblasts and keratinocytes (Schaudies et al., 1985; Wiley et al., 1989; Nickoloff and Mitra, 1989; Khandke et al., 1991) and demonstrate that EGF-receptor down-regulation does not occur in either cell type cultured in basal medium for 48 h. These data therefore rule out the possibility that the lack of EGF/TGF- α -induced PtdIns turnover is due to down-regulation of EGF receptors.

An alternative explanation for the lack of PtdIns turnover in response to EGF/TGF- α in fibroblasts and keratinocytes is that EGF receptors may have become desensitized after culture in basal medium for 48 h. In order to examine this possibility, fibroblasts and keratinocytes, cultured in basal medium for 48 h, were treated with TGF- α (50 nM) for 2–5 min and the cell lysates were immunoblotted and probed with anti-phosphotyrosine monoclonal antibodies to measure TGF- α -stimulated tyrosine phosphorylation. Increased phosphotyrosine content of the 170 kDa EGF receptor and several other proteins was observed in response to TGF- α in both fibroblasts and keratinocytes (Figure 3c). EGF and TGF- α induced tyrosine phosphorylation of the 170 kDa EGF receptor to a similar degree in fibroblasts

Figure 2 Kinetics of EGF-, TGF- α -, thrombin- and Lys-bradykinin-stimulated $\text{Ins}(1,4,5)\text{P}_3$ formation in human dermal fibroblasts (a) and keratinocytes (b)

Sub-confluent fibroblasts and keratinocytes were placed in growth-factor-free basal medium for 48 h and then treated with agonists as described in Figure 1. $\text{Ins}(1,4,5)\text{P}_3$ analysis was carried out as described in the Experimental section. Data points represent mean values of four experiments (a) or two experiments (b).

and keratinocytes (Figure 3c, and results not shown). This 170 kDa band co-migrated with the phosphorylated EGF receptor in A431 membranes (results not shown), and was immunoprecipitated by anti-EGF-receptor monoclonal antibodies (Klein et al., 1992), confirming its identity as the EGF receptor. Thus, under culture conditions in which EGF/TGF- α did not induce PtdIns turnover in fibroblasts and keratinocytes, we observed substantial activation of the EGF-receptor tyrosine kinase in response to TGF- α . These data rule out the possibility that desensitization of the EGF receptor accounts for the lack of PtdIns turnover in response to EGF/TGF- α in human fibroblasts and keratinocytes.

Expression and tyrosine phosphorylation of PLC- γ 1 in cultured fibroblasts and keratinocytes

Recent evidence indicates that EGF- and TGF- α -induced PtdIns hydrolysis is catalysed by PLC- γ 1 (Carpenter and Cohen, 1990). PLC- γ 1 binds to the EGF receptor through interaction of SH2 domains with phosphotyrosine residues, and itself undergoes tyrosine phosphorylation, resulting in its activation (Vega et al., 1992). We therefore examined tyrosine phosphorylation of PLC- γ 1 in human fibroblasts and keratinocytes. Immunoblotting of keratinocyte and fibroblast cell lysates with anti-PLC- γ 1 monoclonal antibodies revealed the presence of a 150 kDa band (Figures 4a and 4b), indicating that PLC- γ 1 protein is expressed in both fibroblasts and keratinocytes. This conclusion was further substantiated by immunoprecipitation studies with anti-PLC- γ 1 polyclonal and monoclonal antibodies, which also revealed a 150 kDa band, of approximately equal intensity, in fibroblasts and keratinocytes (Figures 4a and 4b, and results not shown). We next examined whether TGF- α stimulated PLC- γ 1 tyrosine phosphorylation in fibroblasts and keratinocytes. Cell lysates were immunoprecipitated with anti-PLC- γ 1 monoclonal antibodies and then immunoblotted with either anti-phosphotyrosine monoclonal antibodies or anti-PLC- γ 1 monoclonal antibodies. In samples where PLC- γ 1 protein was clearly present, there was no evidence of TGF- α -induced tyrosine phosphorylation of PLC- γ 1 (Figures 4c and 4d) in either fibroblasts or keratinocytes. In A431 cells, however, TGF- α -induced tyrosine phosphorylation

Figure 3 EGF-receptor expression and tyrosine phosphorylation in human keratinocytes and dermal fibroblasts

^{125}I -EGF binding was performed on sub-confluent cultures of fibroblasts (a) and keratinocytes (b), as described in the Experimental section. Scatchard plots are shown in the inserts. The data points represent means of duplicate dishes. (c) Assessment of EGF-receptor tyrosine phosphorylation in fibroblasts (lanes 1 and 2) and keratinocytes (lanes 3 and 4). Cells were placed in basal medium for 48 h before stimulation with TGF- α (50 nM) for 5 min (lanes 1 and 3), or were left untreated (lanes 2 and 4). Cell lysates were electrophoresed in SDS/8% polyacrylamide gels, transferred to nitrocellulose membrane and immunoblotted with anti-phosphotyrosine monoclonal antibody, as described in the Experimental section.

of PLC γ 1 was readily detectable (Figure 4e), as previously reported (Meisenhelder et al., 1989). Thus it is unlikely that methodological factors, such as inadequate inhibition of phosphatase activity in the immunoprecipitate, prevented detection of PLC- γ 1 tyrosine phosphorylation in TGF- α -treated fibroblasts and keratinocytes.

Previous studies have provided evidence that PKC attenuates some aspects of EGF cellular signalling (Wahl and Carpenter, 1988; Hepler et al., 1988; Decker et al., 1990). We therefore hypothesized that if basal PKC activity was significantly inhibiting EGF receptor signal transduction, in fibroblasts and keratinocytes, down-regulation of PKC should allow us to

Figure 4 Expression and tyrosine phosphorylation of PLC- γ 1

Fibroblasts (**a** and **c**), keratinocytes (**b** and **d**) and A431 cells (**e**) were treated with TGF- α (50 nM) for 2 min (lanes 2 and 5), TGF- α (50 nM) for 5 min (lane 6), bryostatin (50 nM) for 18 h and then TGF- α for 5 min (lane 7), or were left untreated (lanes 1, 3, 4 and 8). Cells were then lysed and mixed with $2 \times$ sample buffer (lanes 1 and 2), or immunoprecipitated with anti-PLC- γ 1 antibody (lanes 3–7), as described in the Experimental section. Lane 8: the anti-PLC- γ 1 antibody was omitted. A portion of each sample was immunoblotted with anti-PLC- γ 1 monoclonal antibodies (**a** and **b**) or with anti-phosphotyrosine monoclonal antibody (**c**, **d** and **e**). Arrowhead indicates position of PLC- γ 1.

demonstrate TGF- α -induced PLC- γ 1 tyrosine phosphorylation. To examine this, cells were treated with bryostatin for 18 h, which results in down-regulation of Ca^{2+} -dependent PKC activity by more than 90% (Klein et al., 1992), with only a transient decrease in EGF receptor expression (Sako et al., 1987; Jetten et al., 1989), and, unlike PMA, does not induce keratinocyte differentiation. Bryostatin pretreatment, however, did not result in detectable tyrosine phosphorylation of PLC- γ 1, in response to TGF- α , in fibroblasts or keratinocytes [Figures 4c (lane 7) and 4d (lane 7)]. Thus the coupling of EGF receptor activation to PLC- γ 1 tyrosine phosphorylation does not appear to be regulated by Ca^{2+} -dependent PKC in fibroblasts or keratinocytes. Prolonged treatment with PMA or bryostatin does not, however, down-regulate all Ca^{2+} -independent PKC isoenzymes (Strulovici et al., 1991; Fisher et al., 1993). Thus attenuation of EGF-receptor-mediated PLC- γ 1 phosphorylation by one or more Ca^{2+} -independent PKC isoenzymes cannot be ruled out.

Effect of EGF and TGF- α on PC hydrolysis and PLD activity in cultured keratinocytes

Having found no evidence for coupling of EGF-receptor activation to PtdIns hydrolysis in either skin fibroblasts or keratinocytes, we next examined coupling of EGF-receptor activation to PC hydrolysis. We have previously observed EGF-stimulated PC hydrolysis in cultured human skin fibroblasts (Fisher et al., 1991). In keratinocytes, incubation with PMA, EGF or TGF- α for between 5 and 30 min failed to raise the levels of intracellular or extracellular choline or phosphocholine (results not shown).

To serve as a positive control, keratinocytes were treated with exogenous bacterial PLC (1 unit/ml) for 10 min. As expected, this produced a 9-fold rise in the level of phosphocholine released into the medium, whereas choline and phosphocholine levels within the cell remained unaltered (results not shown). These data suggest that, in contrast with fibroblasts, EGF and/or TGF- α do not induce PC hydrolysis in human keratinocytes.

To assess further the coupling of the EGF receptor to PLD activation, we next treated [^3H]myristic acid-labelled keratinocytes with agonists, in the presence of 0.5% ethanol, and determined [^3H]phosphatidylethanol production. The transfer of an alcohol to the phosphatidyl group of a phospholipid substrate is a unique property of PLD (reviewed by Shukla and Halenda, 1991) and is a more specific and sensitive assay of PLD activity, in intact cells, compared with choline release. No stimulation of PLD activity was observed in keratinocytes after incubation with TGF- α for 15 min or 30 min (results not shown). Increased PLD activity was, however, observed in response to PMA, as reported in other cell types (Shukla and Halenda, 1991). These data indicate that, although PLD activity is expressed in human keratinocytes, it is not stimulated by EGF or TGF- α . This is in contrast with human skin fibroblasts, in which we have previously observed PLD activation in response to EGF (Fisher et al., 1991).

In order to examine whether the observed difference in stimulation of PC hydrolysis by EGF and TGF- α between fibroblasts and keratinocytes was due to a difference in the level of endogenous PLD or PC-PLC activities, we measured *in vitro* PLD and PC-PLC activities in keratinocyte and fibroblasts.

Figure 5 DAG formation in human fibroblasts and keratinocytes

Sub-confluent cultures of fibroblasts (a) and keratinocytes (c) were placed in basal medium for 48 h. Fibroblasts were incubated with EGF (8.2 nM) or thrombin (5 units/ml) and keratinocytes were incubated with TGF- α (50 nM) or exogenous PLC (5 units/ml), for the indicated times. Cellular DAG mass was analysed as described in the Experimental section. Data points represent means of three experiments (a) or means of duplicate determinations which are representative of two independent experiments (c). (b) Sub-confluent cultures of fibroblasts were placed in basal medium for 30 h before labelling with [³H]myristic acid for 16 h. Cells were stimulated with EGF and thrombin for the indicated times, and [³H]DAG was analysed as described in the experimental section. Data points represent means of three experiments.

Under the reaction conditions employed, we detected membrane-associated PLD activity, but not cytosolic PLD or PC-PLC activities, in fibroblasts and keratinocytes. Membrane-associated PLD activity was found in the heavy membrane pellet, and was 0.6 ± 0.2 nmol/h per mg of protein in keratinocytes and 1.3 ± 0.3 nmol/h per mg of protein in fibroblasts. It is unlikely that this 2-fold lower activity in keratinocytes could account for the observed absence of EGF/TGF- α -stimulated PLD activity in these cells.

EGF- and TGF- α -induced formation of DAG in fibroblasts and keratinocytes

We next examined the kinetics of DAG formation in human fibroblasts and keratinocytes stimulated with EGF and/or TGF- α . In fibroblasts, as we have previously observed (Fisher et al., 1991), EGF stimulated a 3-fold rise in DAG mass, which was maximal at 5 min (Figure 5a). For comparison, fibroblasts were treated with thrombin, which we and others have shown to induce rapid hydrolysis of PtdIns (Wright et al., 1988). In contrast with EGF, thrombin induced a biphasic pattern of DAG formation, with an early peak at 20 s and a second peak at 5 min (Figure 5a). Data from several laboratories indicate that the early rise in agonist-induced 1,2-DAG formation is mainly derived from inositol phospholipids, whereas the second, delayed, peak occurs principally from hydrolysis of PC (Wright et al., 1988; Pessin and Raben, 1989; van Blitterswijk et al., 1991). To examine this in human dermal fibroblasts, cells were labelled with [³H]myristic acid, which is predominantly incorporated into

PC, and [³H]DAG formation in response to EGF and thrombin was determined. In contrast with the observed kinetics of DAG mass formation, the kinetics of [³H]DAG formation in response to EGF and thrombin were similar (Figure 5b). The first peak of DAG formation in response to thrombin was not observed, and both thrombin and EGF induced a delayed rise in [³H]DAG (Figure 5b). These data indicate that EGF-induced DAG formation occurs primarily through PC hydrolysis, and provide additional evidence in support of the absence of EGF-induced PtdIns hydrolysis in human dermal fibroblasts.

In contrast, no significant elevation of DAG mass was observed after treatment of keratinocytes with TGF- α (50 nM) for between 1 and 10 min (Figure 5c) or EGF (50 nM; results not shown). In these experiments Lys-bradykinin was utilized as a positive control and induced a 2-fold increase in DAG mass, as previously reported (Talwar et al., 1989, 1990). In addition, no elevation of DAG mass was observed in keratinocytes incubated with TGF- α (50 nM) for up to 30 min, or a lower dose (1.85 nM) of TGF- α (results not shown). These data are consistent with the observed absence of EGF/TGF- α -induced hydrolysis of PtdIns or PC. These data also suggest that EGF and/or TGF- α do not induce DAG formation in human keratinocytes by an alternative mechanism such as phosphatidylethanol hydrolysis or synthesis *de novo*, as has been reported for other agonists and other cell types (Farese et al., 1987; Kiss and Anderson, 1989).

MARCKS protein phosphorylation in fibroblasts and keratinocytes

The above data indicate that, whereas EGF/TGF- α stimulate PC hydrolysis and DAG formation in dermal fibroblasts neither response occurs in keratinocytes. Since PKC is activated by DAG, this suggests that EGF/TGF- α may activate PKC in dermal fibroblasts, but not in keratinocytes. To assess this directly, we measured EGF- and TGF- α -induced phosphorylation of MARCKS protein, a well-characterized cellular PKC substrate with an apparent molecular mass on SDS/PAGE of 80–87 kDa (Graff et al., 1989). Initially, we characterized the phosphorylation of MARCKS in response to PMA and confirmed its identity as a 80 kDa protein, in fibroblasts and keratinocytes, by immunoprecipitation (Figure 6a). In fibroblasts, EGF induced a substantial increase in MARCKS protein phosphorylation (16-fold compared with control at 2 min, Figures 6b and 6c). The maximum magnitude of EGF-induced phosphorylation was approx. 50% of that observed in response to PMA and occurred between 2 and 5 min after treatment (Figures 6b and 6c). Similar changes were observed with TGF- α (results not shown). In keratinocytes, PMA induced an increase (1.7-fold, $n = 3$) in MARCKS phosphorylation at 10 min ($P < 0.001$ compared with control). Compared with fibroblasts, EGF and TGF- α induced only a modest increase in MARCKS phosphorylation in keratinocytes (1.4-fold at 5 min, $n = 3$, $P = 0.06$, and 1.3-fold at 10 min, $n = 5$, $P = 0.06$, respectively). In order to increase sensitivity and assess whether this was an authentic response, we immunoprecipitated MARCKS in [³²P]_i-labelled agonist-treated keratinocytes. Using this method, a modest but convincing and reproducible increase in MARCKS phosphorylation (1.4-fold at 2 min, compared with 2.5-fold for PMA) in response to TGF- α (50 nM) was detected (Figure 6d). No significant MARCKS phosphorylation was, however, observed in response to lower doses of TGF- α (1.85 nM and 0.185 nM) (results not shown).

These data indicate that in dermal fibroblasts DAG formed through PC hydrolysis in response to EGF and/or TGF- α results in PKC activation. In addition, high doses of TGF- α induces detectable activation of PKC in human keratinocytes,

Figure 6 MARCKS protein phosphorylation in human fibroblasts and keratinocytes

Sub-confluent cultures of fibroblasts and keratinocytes were placed in basal medium for 48 h and then labelled with [32 P]P_i for 3 h, as described in the Experimental section. (a) Fibroblasts (lanes 1 and 2) and keratinocytes (lanes 3 and 4) were stimulated with PMA (50 nM) (lanes 2 and 4) or left untreated (lanes 1 and 3) for 15 min, and immunoprecipitated with an anti-(MARCKS N-terminal peptide) antiserum as described in the Experimental section. The position of the 80 kDa MARCKS protein on the autoradiogram is indicated by the arrowhead. (b) Fibroblasts were incubated with EGF (10 nM) for 0.5 min (lane 2), 1 min (lane 3), 5 min (lane 4), 20 min (lane 5), 25 min (lane 6) or 30 min (lane 7) or with PMA (200 nM) for 1 min (lane 8), or left untreated (lane 1), lysed, heated to 100 °C for 5 min and subjected to SDS/PAGE. The position of the 80 kDa MARCKS protein is indicated by the arrowhead. The autoradiogram was scanned by laser densitometry (c). EGF-induced phosphorylation is expressed in arbitrary units. Data points represent means of three independent experiments. (d) Keratinocytes were treated with TGF- α (50 nM) for 2 min (lane 2), 5 min (lane 3), 10 min (lane 4) or 15 min (lane 5), or with PMA (50 nM) for 2 min (lane 6), 5 min (lane 7), 10 min (lane 8) or 15 min (lane 9), or left untreated (lane 1), lysed and immunoprecipitated with anti-MARCKS antiserum as in (a). (e) Quantification of MARCKS protein phosphorylation in keratinocytes by using a PhosphorImager.

Table 1 TGF- α -induced [3 H]arachidonic acid and [3 H]oleic acid release in human keratinocytes

Sub-confluent cultures of keratinocytes were labelled with [3 H]arachidonic acid or [3 H]oleic acid for 18 h, as described in the Experimental section. Keratinocytes were incubated with TGF- α (50 nM) for the indicated times. [3 H]Arachidonic acid or [3 H]oleic acid released into the medium was determined as described in the Experimental section. Data represent means $\pm$ S.E.M. for triplicate or (*) duplicate determinations and are representative of two independent experiments.

Time (min)	Release (% of control)	
	[3 H]Arachidonic acid	[3 H]Oleic acid
15	169.9 $\pm$ 9.4	105.5 $\pm$ 8.3
30	211.4 $\pm$ 46.7*	124.7 $\pm$ 25.3

but lower doses of TGF- α , which are known to be mitogenic in human keratinocytes, do not significantly activate PKC.

Arachidonic acid release from human keratinocytes

Although TGF- α modestly increased MARCKS protein phosphorylation in human keratinocytes, presumably through activation of PKC, we have consistently been unable to demonstrate TGF- α -induced DAG formation in these cells. EGF activation, in many cells, results in stimulation of phospholipase A₂, and release of arachidonic acid from phospholipids (Clark and Dunlop, 1991, and references therein). Arachidonic acid and other unsaturated fatty acids have been shown to stimulate PKC, in cell-free assays (Shinomura et al., 1991), both alone and synergistically with DAG. Arachidonic acid also enhances PKC activation in intact cells (Yoshida et al., 1992). We therefore investigated whether TGF- α induced release of [3 H]arachidonic acid or [3 H]oleic acid from labelled keratinocytes. Treatment of keratinocytes with TGF- α induced release of [3 H]arachidonic acid 1.7-fold at 15 min, and 2.1-fold at 30 min, compared with control (Table 1). TGF- α -induced release of [3 H]arachidonic acid was relatively selective in that the release of [3 H]oleic acid

from human keratinocytes was significantly less (1.0-fold at 15 min and 1.2-fold at 30 min compared with control; Table 1).

DISCUSSION

The absence of stimulation by EGF or TGF- α of PLC- γ 1 phosphorylation and PtdIns turnover in normal human dermal fibroblasts and keratinocytes is in contrast with an extensive and detailed literature describing the activation of PLC- γ 1 by EGF in other cell types. Most of those latter studies have been conducted with cells that over-express the EGF receptor, i.e. transformed cell lines (e.g. A431 cells) or cells transfected with EGF receptors. EGF is, however, growth-inhibitory to A431 cells in monolayer culture, in which it stimulates PtdIns hydrolysis (Gill and Lazar, 1981). Furthermore, deletional studies with the PDGF receptor suggest that PtdIns hydrolysis is not sufficient for mitogenesis (Escobedo and Williams, 1988; Morrison et al., 1990). Moreover, a specific point mutation in fibroblast growth factor (FGF) receptors abrogated FGF-induced PLC- γ 1 phosphorylation and PtdIns hydrolysis without affecting the mitogenic effect of FGF (Peters et al., 1992; Mohammadi et al., 1992). These studies suggest that PLC- γ 1 phosphorylation and PtdIns hydrolysis may not be required for cell growth induced by activation of tyrosine kinase receptors.

The observed differences in PC hydrolysis between skin fibroblasts and keratinocytes suggests a possible mechanism for diversity of response to EGF/TGF- α among different cell types. The lack of PtdIns hydrolysis, PC hydrolysis or DAG formation, in response to EGF or TGF- α in keratinocytes, indicates that EGF receptor activation is not coupled to PtdIns-PLC, PC-PLC or PLD in these cells. We observed only a modest increase in MARCKS phosphorylation in response to TGF- α (50 nM) in keratinocytes. Furthermore, no significant activation of PKC was observed in response to lower doses of TGF- α (1 and 10 ng/ml) that are known to be mitogenic. Therefore, the well-characterized mitogenic effect of EGF and TGF- α in keratinocytes is likely to be independent of PKC activation. These data are consistent with the participation of PKC in keratinocyte terminal differentiation. Activation of PKC by PMA in keratino-

cytes leads to growth arrest and induction of phenotypic differentiation, i.e. cell cornification (Hawley-Nelson et al., 1982). There is no evidence that, in keratinocytes, down-regulation of PKC activity by bryostatin leads to potentiation of EGF receptor kinase activity or enhanced TGF- α autoinduction (Klein et al., 1992). In human dermal fibroblasts, which do not undergo phenotypic differentiation or growth in response to PMA, PKC may serve, among other things, to act as a negative regulator of EGF-receptor activation, as demonstrated in other cell types (Wahl and Carpenter, 1988; Hepler et al., 1988; Decker et al., 1990).

Finally, our data suggest that in skin fibroblasts DAG formed through PC hydrolysis is able to activate PKC. The observed time courses of EGF-induced DAG elevation and MARCKS phosphorylation are consistent with this conclusion. In contrast, Leach et al. (1991) failed to observe PKC activation in the presence of increased DAG formed through PC hydrolysis, and prolonged treatment of Swiss 3T3 fibroblasts with PMA did not inhibit the mitogenic effect of exogenous *Bacillus cereus* PC-PLC, suggesting that the mitogenic effect of PC-PLC was independent of PKC activation (Larrodera et al., 1990). These differences presumably reflect differences among cell types, perhaps in expression of PKC isoenzymes. IIC9 fibroblasts express α , but not β or γ , PKC isoforms (Leach et al., 1991). The expression of non-conventional PKC isoforms, however, has not been defined in these cells. Human dermal fibroblasts express the δ , ϵ and ζ PKC isoforms in addition to PKC- α (Fisher et al., 1993), and this may account for the observed differences between these cell types.

Why EGF and/or TGF- α stimulates PC hydrolysis in human dermal fibroblasts, but not in keratinocytes, remains to be elucidated. Fibroblasts and keratinocytes express similar levels of high-affinity EGF receptors and membrane-associated PLD activities. EGF and TGF- α induce EGF-receptor tyrosine phosphorylation in fibroblasts and keratinocytes to a similar degree. These data suggest that coupling of EGF receptor to PC hydrolysis is different in fibroblasts and keratinocytes.

In summary, three major conclusions emerge from our studies: (1) in two normal cell types from human skin, EGF receptor activation is not coupled to stimulation of PLC- γ 1 or PtdIns hydrolysis, (2) coupling of EGF-receptor activation to PC turnover is cell-type specific, and (3) DAG formed from PC hydrolysis is capable of activating PKC, in skin fibroblasts.

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