Second messenger modulation of epidermal growth factor receptor function does not occur at the level of receptor dimerization

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Epidermal growth factor (EGF)-induced receptor dimerization may provide a mechanism for activation of the receptor protein tyrosine kinase and for initiation of post-receptor signalling pathways. We have examined whether second messengers and agents that modulate EGF receptor function act at the level of receptor dimerization. Both the Ca^{2+} ionophore ionomycin and the tumour promotor tetradecanoylphorbol acetate (TPA), added shortly before EGF, inhibit EGF receptor protein tyrosine kinase activity in intact cells. In permeabilized cells, elevation of Ca^{2+} similarly inhibits EGF receptor function. The inhibitory effect of Ca^{2+} , unlike that of TPA, appears not to be dependent on protein kinase C activity. Neither ionomycin nor phorbol ester affects EGF-induced receptor dimerization, as shown by cross-linking and immunoblotting techniques, although the phosphotyrosine content of both monomeric and dimeric receptors is strongly decreased. Furthermore, we show that EGF receptor dimerization is not affected by increases in cyclic AMP or intracellular pH, nor by changes in transmembrane potential, medium osmolarity or the glycosylation state of the receptor. These results suggest that modulation of EGF receptor function occurs at a step other than receptor dimerization.

INTRODUCTION

The epidermal growth factor (EGF) receptor is a single-chain transmembrane glycoprotein with ligand-dependent protein tyrosine kinase activity [1]. Activation of the receptor tyrosine kinase is essential, although not necessarily sufficient, for the multiple biological effects of EGF [2,3]. Early events induced by EGF include tyrosine-specific protein phosphorylations, inositol lipid breakdown, changes in cytoplasmic pH and free Ca^{2+} and alterations in membrane potential [1–7]. To explain how EGF binding results in activation of the intracellular kinase domain, a model has been proposed in which EGF promotes the formation of receptor homodimers [8,9]. The dimeric state would provide a conformation that favours receptor activation, resulting in receptor autophosphorylation, substrate phosphorylation and signal transduction (see [9] for review). EGF-induced receptor dimerization has been detected both in purified receptor preparations and in intact cells [10,11]. The model is further supported by the observation that purified EGF receptor dimers exhibit increased tyrosine kinase activity when compared with receptor monomers [12]. In addition, recent evidence suggests that autophosphorylation of solubilized EGF receptors is mediated, at least in part, by intermolecular cross-phosphorylation, probably as a result of receptor oligomerization [13].

Various agents, in particular phorbol esters and second messengers such as Ca^{2+} and cyclic AMP, are known to affect EGF receptor activity and to modulate signal transduction. Perhaps the best-documented mechanism of regulation is via protein kinase C. The EGF receptor is phosphorylated on threonine and serine residues by protein kinase C, resulting in an inhibition of tyrosine kinase activity of the receptor [14–18]. EGF can mimic phorbol esters in stimulating phosphorylation of residue Thr-654, presumably as a result of increased diacylglycerol production and consequent activation of protein kinase C [19,20]. Furthermore, down-regulation of protein kinase C leads to enhanced EGF receptor kinase activity in A431 cells [21].

Taken together, the available data suggest feedback regulation of EGF receptor functions via protein kinase C.

Among the earliest detectable events in EGF action is a rapid increase in intracellular $[\text{Ca}^{2+}]$ [2–4], which may lead to activation of a Ca^{2+} /calmodulin-dependent protein kinase and contribute to activation of protein kinase C, while artificially raising intracellular $[\text{Ca}^{2+}]$ affects high-affinity EGF binding and endocytosis and decreases receptor kinase activity [22–24].

Another means of EGF receptor regulation may involve cyclic AMP. EGF receptors can be phosphorylated by cyclic AMP-dependent protein kinase *in vitro* [25], and elevation of intracellular cyclic AMP levels has been reported to decrease binding affinity for EGF [26].

It is not clear at what level regulation of EGF receptor kinase activity and signal transduction occurs. It seems plausible to assume that receptor dimerization provides a mechanism not only for triggering but also for modulating receptor kinase activity. In the present study, we have tested this hypothesis by using covalent cross-linkers and immunoblotting techniques. The results indicate that receptor dimerization in intact cells is not inhibited by raising intracellular Ca^{2+} levels or by activation of protein kinase C, although tyrosine kinase activity is markedly lowered. Furthermore, dimerization appears not to be affected by manipulating other parameters involved in EGF receptor signal transduction, such as intracellular cyclic AMP, pH and transmembrane potential. The present data strongly support the notion that regulation of EGF receptor function is secondary to the dimerization process.

MATERIALS AND METHODS

Materials

EGF was obtained from Collaborative Research Inc. The covalent cross-linking agents EDAC [1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide], DSS (disuccinimidyl suberate) and

Abbreviations used: EGF, epidermal growth factor; TPA, tetradecanoylphorbol acetate; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide; DSS, disuccinimidyl suberate; BS³, bis(sulphosuccinimidyl) suberate; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; DMM, 1-deoxymannojirimycin.

BS³ [bis(sulfosuccinimidyl) suberate] were from Pierce; ¹²⁵I-labelled EGF and myo-[³H]inositol were from Amersham.

Cells

Human epidermoid carcinoma cells (A431) and mouse NIH-3T3 cells transfected with the human EGF receptor (expressing approx. 300 000 receptors per cell; kindly provided by J. Schlessinger, New York University Medical Center) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum.

EGF receptor cross-linking in intact cells

Cells were grown to near-confluency in 35 mm diameter dishes and washed three times in phosphate-buffered saline (PBS; 137 mM-NaCl, 3 mM-KCl, 1 mM-CaCl₂, 0.5 mM-MgCl₂, 8.1 mM-Na₂HPO₄, 1.5 mM-KH₂PO₄) or assay buffer (120 mM-NaCl, 6 mM-KCl, 1.2 mM-MgCl₂, 1 mM-CaCl₂ and 25 mM-Hepes, pH 7.5) containing 30 µM-BSA; BSA was omitted in experiments with ionomycin and monensin. After preincubation with various agents and addition of EGF, incubations were continued in the presence of cross-linker for various periods of time. Stock solutions of cross-linkers (100 mM-EDAC in PBS, 100 mM-BS³ in assay buffer or 25 mM-DSS in dimethyl sulfoxide) were made immediately before addition to the cells. Cross-linking with EDAC (10 mM) was carried out at 37 °C and with BS³ (2 mM) and DSS (0.2 mM) on ice. DSS was used in the majority of the experiments. After 15 min, cross-linking was stopped by washing the cells three times with Ca²⁺-free PBS containing 50 mM-glycine, 50 mM-Tris and 4 mM-EDTA. Cells were lysed in sample buffer (3% SDS, 25 mM-glycine, 10% glycerol, 50 µM-sodium vanadate and 10 mM-*p*-nitrophenyl phosphate), boiled for 5 min and sonicated for 5 s. After duplicate protein determinations according to the method of Lowry *et al.* [27], equal amounts of protein were loaded on either 5% or 3–10% linear polyacrylamide gels for SDS/PAGE. Following electrophoresis, proteins were transferred to nitrocellulose. The remaining sites were blocked with BSA (3%) in TBST (10 mM-Tris/HCl, pH 8.0, 150 mM-NaCl and 0.05% Tween-20) and blots were probed with an anti-(EGF receptor) antibody 281-7, raised against the peptide sequence 984–996 [28], or with a monoclonal anti-phosphotyrosine antibody [29] followed by incubation with rabbit anti-mouse serum (Nordic, Tilburg, The Netherlands). Antibody binding was detected using ¹²⁵I-Protein A and autoradiography.

Dimerization of purified EGF receptors

EGF receptor was purified from A431 cells, essentially as described by Cochet *et al.* [11]. A431 cells (3 × 10⁷) were washed three times with PBS and lysed in lysis buffer (0.5% Nonidet P-40, 5 mM-MgCl₂, 50 mM-Tris, pH 7.4, 1 mM-phenylmethanesulphonyl fluoride, 10 µg of leupeptin/ml and 0.3 trypsin inhibitory units of aprotinin/ml). Cellular debris was removed by centrifugation and the supernatant was extensively precleared with normal mouse serum coupled to Protein A-Sepharose beads. Anti-(EGF receptor) monoclonal antibody 108 [13] covalently coupled to Protein A-Sepharose beads was added and the suspension was stirred for 2 h on ice. The beads were washed three times with HTG buffer (20 mM-Hepes, pH 7.5, 0.1% Triton X-100 and 10% glycerol), twice with borate buffer (50 mM-borate, pH 8.0, 0.5 M-NaCl, 0.3 M-MgCl₂, 10% glycerol and 0.1% Triton X-100) and twice more with HTG buffer. EGF receptors were eluted with 50 mM-glycine/HCl (pH 2.5)/10% glycerol/0.1% Triton X-100 and the eluate was immediately neutralized with 0.1 M-NaOH and 600 mM-Hepes (pH 7.5). EGF was added to part of the material obtained and after stirring for 20 min at room temperature freshly dissolved DSS was added to

a final concentration of 0.2 mM. After 30 min cross-linking was stopped and samples were prepared for electrophoresis and transferred to nitrocellulose. Ponceau S staining of the nitrocellulose revealed one band of about 170 kDa without major contaminants. Immunoprecipitation of lysates of A431 cells labelled with [³⁵S]methionine revealed only some minor low-molecular-mass contaminants (results not shown). Immunodetection of the receptor monomers, dimers and trimers was done as described for cell lysates.

Permeabilization with streptolysin-O

A431 cells were permeabilized essentially as described by Tilly *et al.* [30]. Briefly, confluent monolayers were washed in permeabilization buffer (137 mM-KCl, 2 mM-MgCl₂, 1 mM-NaATP and 5 mM-Pipes, pH 6.8) and then incubated in permeabilization buffer containing 0.4 units of streptolysin-O/ml for 10 min. Permeabilization was monitored by Trypan Blue uptake (> 90% leaky). After washing the permeabilized cells three times with incubation buffer (130 mM-KCl, 10 mM-NaCl, 2 mM-MgCl₂, 1 mM-NaATP and 5 mM-Pipes, pH 6.8), compounds were added in the incubation buffer as described in the Figure legends.

RESULTS

Covalent cross-linking *in vivo* and *in vitro*

EGF receptor dimerization *in vivo* and *in vitro* was studied using various covalent cross-linking agents. By using SDS/PAGE and immunoblotting we were able to monitor EGF-induced receptor dimerization in A431 cells, transfected NIH 3T3 cells and HeLa cells, expressing about 2 × 10⁶, 3 × 10⁶ and 3 × 10⁴ human EGF receptors/cell respectively. A typical dimerization

Fig. 1. EGF receptor dimerization in NIH-3T3 cells

Transfected NIH-3T3 cells were incubated with 0–500 ng of EGF/ml in PBS at 37 °C. After 5 min, incubations were continued in the presence (+) or absence (–) of 10 mM-EDAC; 15 min later, cross-linking was stopped and cell lysates were run on a 5% polyacrylamide gel for SDS/PAGE. EGF receptor monomers (170 kDa, ◀) and dimers (360 kDa, ◀◀) were detected by immunoblotting with anti-peptide antibody 281-7, followed by labelling with ¹²⁵I-Protein A. Molecular mass markers: myosin, 200 kDa; phosphorylase B, 104 kDa. Similar results were obtained in A431 and HeLa cells.

Fig. 2. Comparison of dimerization *in vivo* and *in vitro*

Lanes 1–4: A431 cells were incubated with (+) or without (–) 100 ng of EGF/ml and after 5 min at 37 °C 0.2 mM-DSS (+) or dimethyl sulphoxide (–) was added. After cross-linking on ice for 15 min, cell lysates were prepared as described. Lanes 5 and 6: EGF receptor purification from A431 cells and receptor dimerization were performed as described in the Materials and methods section. EGF was added at 1 µg/ml and cross-linking was done at room temperature. The blot was developed with anti-peptide serum 281–7.

experiment with transfected NIH-3T3 cells using EDAC (10 mM) as a covalent cross-linker is shown in Fig. 1. In the immunoblot, a high-molecular-mass band of about 360 kDa, corresponding to the molecular mass of EGF receptor homodimers, is clearly detectable when cells were treated with both EGF and EDAC. The intensity of this band varied between different experiments but was always maximal at 100–200 ng of EGF/ml, as confirmed by counting the radioactivity in the excised bands (results not shown). The use of other bifunctional cross-linkers such as DSS and BS³ yielded similar results.

The appearance of the 360 kDa band shown here and by others [11,31–33] is most readily explained in terms of the induction of receptor homodimers. However, it remains formally possible that the receptor is cross-linked to a heterologous membrane protein of similar molecular mass. To investigate this point in further detail we monitored EGF receptor dimerization in purified receptor preparations. Fig. 2 shows that the EGF-induced 360 kDa band of intact A431 cells co-migrates with the dimer band of purified EGF receptors, suggesting that the 360 kDa band in intact cells indeed represents EGF receptor dimers. In the purified receptor preparation some spontaneous dimer formation is detectable in the absence of EGF, which is not the case in intact cells. Importantly, dimer formation of isolated receptors occurs in the absence of ATP, supporting the view that receptor tyrosine kinase activity is not essential for receptor dimerization. We further note that EGF also evokes the appearance of a band of approx. 520 kDa in purified receptors (but not usually in intact cells), presumably representing receptor trimers (Fig. 2).

Effect of elevated intracellular Ca²⁺

EGF evokes a rapid increase in cytoplasmic free Ca²⁺ in its target cells [2–4]. Ca²⁺ functions as a second messenger that regulates various cellular functions. In particular, raising intracellular [Ca²⁺] with an ionophore modulates EGF receptor

Fig. 3. Effect of intracellular [Ca²⁺] on EGF receptor dimerization and autophosphorylation

A431 cells were preincubated for 3 min with 4 mM-EGTA (E), 5 µg of ionomycin/ml (I) or both (I + E), or with dimethyl sulphoxide as a control (C). EGF (100 ng/ml) was added except for lane 1 (–), and 5 min later incubations were continued in the presence of 0.2 mM-DSS at 0 °C. Samples prepared for immunoblotting were divided into two. The immunoblots were developed with either anti-peptide antibody 281–7 (a) or anti-phosphotyrosine antibody (b) and ¹²⁵I-Protein A.

binding, endocytosis and tyrosine kinase activity of the receptor [16,22–24]. Hence Ca²⁺ is a potential candidate for feedback regulation of EGF receptor functions.

We investigated whether an elevated Ca²⁺ level is capable of modulating EGF receptor dimerization. The Ca²⁺-ionophore ionomycin was added shortly before addition of EGF both in the presence and the absence of EGTA. As shown in Fig. 3, ionomycin did not interfere with EGF receptor dimerization, whereas EGF-induced receptor autophosphorylation is seen to be almost completely abolished. Furthermore, phosphorylation of several endogenous substrates was also inhibited by ionomycin (results not shown). However, when added in the presence of EGTA to prevent Ca²⁺ influx, ionomycin had no detectable effect on the phosphorylation state of the EGF receptor (Fig. 3).

Studies in permeabilized cells

To examine further the effect of Ca²⁺ on EGF receptor function, A431 cells were permeabilized with streptolysin-O and tested for EGF receptor autophosphorylation in the presence or absence of Ca²⁺. Fig. 4 shows a dramatic decrease in EGF-induced tyrosine kinase activity when Ca²⁺ was present (1 mM), but not in nominally Ca²⁺-free media. Ionomycin alone had no detectable effect in permeabilized cells (Fig. 4). A Ca²⁺-sensitive proteinase has been described [34] that degrades the EGF receptor into a doublet of 150 kDa and 170 kDa. As is seen in the immunoblots shown in Fig. 4, the receptor was not cleaved into such a doublet, nor was a 20 kDa breakdown product observed in any of our experiments (results not shown). Thus the decrease in EGF receptor kinase activity caused by Ca²⁺ cannot be due to activation of the Ca²⁺-dependent proteinase.

Effect of phorbol ester

Brief treatment of cells with tetradecanoylphorbol acetate (TPA) is known to result in significant inhibition of EGF receptor kinase activity [14,16,17]. To determine whether inhibition of EGF receptor activity by protein kinase C correlates with altered receptor dimerization, cells were briefly treated with

Fig. 4. Effect of Ca²⁺ on EGF-induced receptor kinase activity in A431 cells permeabilized with streptolysin-O

A431 cells were permeabilized as described in the Materials and methods section, and EGTA (4 mM), Ca²⁺ (1 mM) and ionomycin (5 µg/ml) were added as indicated. EGF (100 ng/ml) was added and incubations were continued for 5 min at 37 °C and then for 15 min at 0 °C. Cells were lysed and SDS/PAGE and immunoblotting were performed using anti-phosphotyrosine antibody and ¹²⁵I-Protein A.

TPA, and receptor dimerization and autophosphorylation were monitored by immunoblotting (Fig. 5). At each EGF concentration (0–100 ng/ml), the intensity of both monomeric and dimeric receptor bands in TPA-treated cells was not significantly different from that in untreated cells (Fig. 5a). However, when the blot was probed with anti-phosphotyrosine antibody, TPA treatment was found to result in a marked decrease in the intensity of both monomer and dimer bands (Fig. 5b). The results indicate that, although activation of protein kinase C by TPA inhibits EGF-induced tyrosine kinase activity *in vivo*, EGF receptor dimerization is not affected. We conclude that the

Fig. 5. Effect of TPA on EGF receptor dimerization and autophosphorylation

A431 cells were pretreated with 100 ng of TPA/ml (+) or with dimethyl sulphoxide (–) for 15 min at 37 °C. Incubations were continued on ice in the presence of 0–100 ng of EGF/ml. After 3 h, 0.2 mM-DSS was added and 15 min later samples were prepared for immunoblotting. The immunoblot was first developed with anti-phosphotyrosine antibody and ¹²⁵I-Protein A (b). Then the blot was acid-washed (pH 2.5) and reprobed with antibody 281–7 and ¹²⁵I-Protein A (a). ◀, 170 kDa; ◀, 360 kDa.

Fig. 6. Effect of ionomycin on EGF-induced kinase activity in protein kinase C down-regulated cells

A431 cells were cultured for 72 h in the presence of (a) 0.01 % dimethyl sulphoxide (control) or (b) 500 ng of TPA/ml. After thoroughly washing the cells, TPA (T, 100 ng/ml), EGTA (E, 4 mM) or ionomycin (I, 5 µg/ml) were added at 15, 5 and 3 min, respectively before addition of EGF (100 ng/ml). After 5 min at 37 °C, incubations were continued at 0 °C for another 15 min. Thereafter, samples were prepared for immunoblotting with anti-phosphotyrosine antibody.

inhibitory action of protein kinase C on EGF receptor signal transduction does not occur at the level of dimerization.

Studies in protein kinase C down-regulated cells

At high Ca²⁺ concentrations protein kinase C may be activated without the requirement for diacylglycerol or phorbol ester. To examine whether the observed effects of ionomycin/Ca²⁺ are mediated by protein kinase C, A431 cells were treated for 72 h with phorbol ester (0.5 µg/ml) to down-regulate functional protein kinase C activity. Indeed, such chronic treatment of the cells with TPA leads to complete loss of [³H]phorbol dibutyrate binding sites and failure of TPA to induce phosphorylation of the 80 kDa protein kinase C substrate (T. Eicholtz & G. F. Verheijden, unpublished work). Fig. 6 shows that TPA failed to lower EGF receptor autophosphorylation in such kinase C-depleted cells. In marked contrast with TPA, ionomycin was still capable of abolishing receptor kinase activity when protein kinase C was down-regulated. Taken together, these results indicate that the inhibitory effect of Ca²⁺ on EGF receptor function is independent of functional protein kinase C.

Effects of cyclic AMP, pH, membrane potential and hyperosmolarity

To determine whether increases in cyclic AMP and pH can modulate EGF receptor dimerization, cells were treated with the cell-permeant cyclic AMP analogue 8-bromo-cyclic AMP and the Na⁺/H⁺ ionophore monensin respectively. As shown in Fig. 7, neither treatment affected receptor dimerization.

EGF has been reported to transiently alter the membrane potential in cells of epithelial origin [5,7]. It is conceivable that the electric field across the plasma membrane may act on charged residues of the receptor located near the inner side of the plasma membrane or that it may modulate putative non-covalent interactions that could be important for dimer formation. To test the possible effects of alterations in transmembrane potential, dimerization was monitored in cells incubated in medium containing 150 mM-K⁺, which abolishes the transmembrane potential. Fig. 7(a) shows that dimerization *in vivo* in this

Fig. 7. Effect of various agents on the dimeric state of the EGF receptor

(a) A431 cells were treated with 8-bromo-cyclic AMP (0.5 mM) (A) for 10 min. EGF (100 ng/ml) was added and 5 min later cross-linking was carried out at 37 °C using DSS (0.2 mM). C represents untreated control cells. (b) A431 cells were incubated in high-K⁺ (150 mM) buffer (K), monensin (10 μM)-containing buffer (M) or hyperosmotic buffer (N) to which 100 mM-NaCl was added. C represents untreated control cells. Preparation of cell lysates and immunoblotting with antibody 281-7 was carried out as described in the Materials and methods section. ◀, 170 kDa; ◀, 360 kDa.

high-K⁺ buffer was comparable with the control, indicating that the electric field across the plasma membrane is not important for receptor dimerization.

In a recent study the EGF receptor was found to be phosphorylated by an unknown mechanism, not involving protein kinase C, when A431 cells were exposed to a hyperosmotic shock [19]. In addition, we have observed that hyperosmotic shock, induced by adding 100 mM-NaCl to the incubation medium, inhibits EGF-induced inositol phosphate formation (G. F. Verheijden, I. Verlaan & W. H. Moolenaar, unpublished work). Fig. 7(b) shows that dimerization under these conditions is comparable with that in the control, indicating that hyperosmolarity does not affect EGF-induced receptor dimerization.

Effect of treatment with glycosylation inhibitors and neuraminidase

The mature EGF receptor (170 kDa) contains a relatively large amount of *N*-linked oligosaccharides [1,35–37], but whether the glycosylation state of the receptor has a role in dimerization is not known. Addition of core oligosaccharides to the peptide backbone (130 kDa) is essential for EGF binding, but terminal processing of the carbohydrate moiety is not. We tested the effects of three different agents that interfere with carbohydrate processing on EGF receptor dimerization. We used (1) swainsonine, an inhibitor of *cis*-Golgi α -mannosidase II, which blocks normal processing of high mannose chains to complex forms but leaves EGF binding almost unaffected [35], (2) 1-deoxymannojirimycin (DMM), which inhibits mannosidase I in the rough endoplasmic reticulum [38], and (3) neuraminidase, which removes sialic acids. As shown in Fig. 8, EGF-induced receptor dimerization was clearly detectable after pretreatment of A431 cells with DMM, swainsonine or neuraminidase, whereas the apparent molecular mass of both monomers and dimers was slightly decreased, most probably as a result of changes in the carbohydrate moiety. Similar results were obtained when the

Fig. 8. Effect of altered *N*-linked glycosylation on EGF receptor dimerization

(a) A431 cells were cultured for 44 h in the absence (C) or presence of 1 μg of swainsonine/ml (S) or 1 mM-DMM (D). Cells were washed and after 5 min of incubation in the presence (+) or absence (–) of EGF (100 ng/ml), 2 mM-BS³ was added to the medium. Cell lysates were prepared and the immunoblot was developed with antibody 281-7. (b) A431 cells were treated with 5 units of neuraminidase/ml (N) for 30 min on ice. C represents control. The experiment was continued as described for (a). ◀, 170 kDa; ◀, 360 kDa.

blot was probed with an anti-phosphotyrosine antibody, indicating that the EGF-induced tyrosine kinase activity was not affected (results not shown). Together, these data indicate that terminal processing of *N*-linked oligosaccharides is not essential for EGF receptor dimerization.

DISCUSSION

The EGF receptor is the prototype of the family of growth factor receptors with intrinsic tyrosine kinase activity (see [1,39] for reviews). The ligand-binding domain and the tyrosine kinase domain of these receptors are connected by a single transmembrane spanning stretch of amino acid residues. There are two contrasting views regarding the mechanism of ligand-induced receptor activation. An intramolecular mechanism of receptor activation would require that ligand-induced conformational changes in the extracellular domain are transmitted to the intracellular kinase domain through this single transmembrane spanning region. Evidence has been presented that such an intramolecular mechanism of EGF receptor kinase activation may indeed occur under certain experimental conditions [40,41]. On the other hand, Schlessinger [9] has proposed an intermolecular mechanism of receptor activation. According to the latter model, EGF stabilizes its receptor in a dimeric conformation, thereby facilitating protein–protein interactions between one or more domains and leading to activation of the receptor tyrosine kinase. Our present data, together with previously published results, provide strong support for this dimerization model.

Treatment of intact cells with EGF causes the appearance of a band of approx. 360 kDa, consistent with the formation of

EGF receptor homodimers, accompanied by increased tyrosine autophosphorylation of both monomers and dimers in a concentration- and time-dependent fashion. Our results and those of others leave open the possibility that the EGF-induced 360 kDa band represents pre-existing EGF receptor dimers which can only be trapped by cross-linking upon binding of EGF. On the other hand, since the 360 kDa band seen in intact cells has not yet been formally proven to represent EGF receptor homodimers, this band could conceivably reflect dimer formation between EGF receptors and heterologous molecules with similar electrophoretic mobility. The mechanisms that control ligand-induced receptor dimerization are still unresolved. In particular, it remains unclear whether modulation of EGF receptor function and signal transduction by second messengers such as Ca^{2+} , diacylglycerol and cyclic AMP may occur at the level of receptor dimer formation. Conversely, it is of obvious importance to determine whether experimental protocols can be developed that inhibit EGF receptor dimer formation, and, if so, whether post-receptor signalling is similarly affected.

In the present study we have used covalent cross-linkers and immunoblotting protocols to monitor receptor dimerization in intact cells. Since covalent cross-linking *in vivo* is not a highly efficient process, resulting in variable yields in different experiments, the actual amount of dimers formed may be underestimated. Nevertheless, quantitative changes in the receptor aggregation state can be assessed by comparing the intensities of the bands of treated cells to those of untreated cells in individual experiments. In separate experiments (results not shown), we have applied native protein separation techniques, such as non-denaturing gel electrophoresis and sucrose density gradients, in an attempt to circumvent the use of cross-linkers; however, although these alternative approaches readily allowed detection of dimers in purified receptor preparations, we failed to observe dimer formation *in vivo* without the use of covalent cross-linkers.

We show here that EGF receptor dimerization is not inhibited by elevated Ca^{2+} levels or by TPA-activated protein kinase C under conditions where the phosphotyrosine content of both monomeric and dimeric receptors is drastically decreased. Hence inhibition of EGF receptor kinase activity by Ca^{2+} or TPA must occur independently of receptor dimerization. Our findings obtained with TPA are supported by a recent report by Northwood & Davis [32], who used a different procedure to detect the dimeric state of receptor. Although the inhibitory effect of Ca^{2+} ionophore on EGF receptor autophosphorylation is similar to that of TPA, suggesting involvement of protein kinase C, our own data obtained from protein kinase C-depleted cells (Fig. 6) and those of others [24] indicate that Ca^{2+} does not act through protein kinase C. It can also be ruled out that Ca^{2+} -dependent proteinases have a role in inhibiting kinase activity. It seems plausible to assume that Ca^{2+} acts through Ca^{2+} /calmodulin kinase that may phosphorylate the EGF receptor, but formal proof for this hypothesis is lacking at present.

Furthermore, we have shown that alterations in cyclic AMP and cytoplasmic pH, medium osmolarity and transmembrane potential do not appear to affect the dimerization process in a significant manner. Finally, we have modified terminal processing of N-linked oligosaccharides by various metabolic inhibitors of carbohydrate processing, but receptor dimerization is not detectably affected by these agents. In conclusion, our results strongly suggest that modulation of EGF receptor function and post-receptor signalling occurs at a step distal to receptor dimerization.

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