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Cholesterol and phosphoinositides increase affinity of the epidermal growth factor receptor

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The epidermal growth factor receptor (EGF-R) has been purified from human epidermoid carcinoma A431 cells by affinity chromatography in a single step using a monoclonal antibody (528) which competes with EGF for receptor binding. The purified EGF-R exhibits EGF inducible tyrosine kinase and autophosphorylation activity. Steady-state binding of EGF to the purified receptor revealed the presence of one class of binding sites exhibiting an apparent dissociation constant (K_d) of approx. 2 nM. When Angiotensin II was used as a receptor tyrosine kinase substrate the specific activity of the EGF induced kinase was 87 nmol/min per mg and the K_m of the reaction was about 2 mM. Reconstitution of the EGF receptors into lipid vesicles was achieved by octylglucoside dialysis. Reconstitution of the receptor into pure dioleoylphosphatidylcholine (DOPC) vesicles had no effect on the EGF-binding properties in comparison to receptors in Triton X-100 micelles. Binding of EGF to the reconstituted receptor with ATP and Angiotensin II incorporated into the vesicles resulted in a five fold stimulation of the receptor kinase activity. The introduction of cholesterol, ranging from 10% to 50% (w/w), into DOPC vesicles resulted in an increase of the affinity of the receptor for its ligand. The K_d for EGF decreased from 1.8 nM in pure DOPC vesicles to 0.3 nM in DOPC/cholesterol (1:1 (w/w)) vesicles. With the introduction of small amounts (2% (w/w)) of phosphatidylinositol lipids into DOPC vesicles the K_d changed from 1.8 nM to 0.2 nM with phosphatidylinositol (PtdIns) and phosphatidylinositol 4,5-biphosphate (PtdIns4,5-P_2) and to 0.1 nM in the case of phosphatidylinositol 4 phosphate (PtdIns4-P). No change in affinity was found when equal amounts of phosphatidylserine (PS) or phosphatidic acid (PA) were used.

Introduction

Phospholipids and cholesterol are building blocks of biomembranes forming a fluid bilayer continuum between the membrane proteins. In this concept the role of lipids is simply to separate two aqueous compartments and to guarantee a semi-permeable barrier. In addition to this general property crucial roles can be attributed to particular lipid classes during biological processes, polyphosphoinositides [1,2] and diacylglycerol [3–5] in signal transduction, and glycolipids in receptor functioning [6].

Phospholipids or its derivates may act as activators for enzymes involved in signal transduction, the best known example being the activation of protein kinase C (PKC) by diacylglycerol. Phospholipids may be required for enzyme activity in general, such as the dependency of PKC activity on the presence of PS [7].

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In addition a number of studies indicate that phospholipids may influence receptor induced signal transduction by directly affecting receptor functioning. It has been demonstrated that binding of insulin to its receptor is dependent upon the lipid composition of the surrounding membrane [8]. The lipid composition does not only influence the binding affinity, but in some cases also the specificity of the receptor, as has been shown for the vitronectin receptor [9]. Vitronectin receptor inserted into PC liposomes binds only vitronectin, but when it was inserted into phosphatidylcholine-phosphatidylethanolamine (PC-PE) and phosphatidylcholine-phosphatidylethanolamine-phosphatidylserine-phosphatidylinositol (PC-PE-PS-PI)cholesterol liposomes it binds both to von Willebrand factor and fibronectin. In many cases the effects of lipids on enzyme activity are caused by modulations of the fluidity of the membrane, by changes of the surface charge of the membrane or by modification of the hydrocarbon chains adjacent to the lipid-protein interface.

In this paper we have studied the effects of lipids on EGF-R binding affinity for its natural ligand (EGF).

For that reason the EGF-R has been isolated and purified using affinity chromatography. The purified receptor was reconstituted into lipid bilayer vesicles by exchanging Triton X-100 with octylglucoside and dialysing the latter detergent in the presence of exogenous added phospholipids. The EGF-binding characteristics and receptor kinase activity were tested before and after reconstitution and the effect of cholesterol and negatively-charged phospholipids in particular phosphoinositides on the binding properties have been investigated.

Materials and Methods

Cell culture. A431 epidermoid carcinoma cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Paisley, UK) supplemented with 7.5% Fetal Calf Serum (FCS; Integro, Zaandam, The Netherlands) in 1750-cm^2 roller bottles (Falcon; Becton Dickinson Labware, UK) to confluency.

EGF-R purification. Cells were washed twice with phosphate-buffered saline (PBS) and detached with PBS containing 1 mM EDTA. The cells were collected by means of centrifugation for 10 min at $800 \times g$ suspended in solubilisation buffer containing 10 mM Tris-HCl (pH 7.4), 1% Triton X-100, 10% glycerol, 150 mM NaCl, 1 mM EDTA, 5 μg/ml leupeptin, 5 mM benzamidin, 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF) and incubated for 1 h on ice. The insoluble material was removed by centrifugation at 100000 $\times g$ for 1 h at 0°C. The clear supernatant was passed through a small column of Sepharose CL-4B (Pharmacia, Uppsala, Sweden) which contained covalently-coupled 528, a monoclonal antibody directed against the external domain of the EGF receptor [10]. The column was subsequently washed with 100 ml of the following buffers: (1) 10 mM Tris-HCl (pH 7.4), 0.1% Triton X-100, 1 M NaCl, 10% glycerol, 1 mM EDTA, 5 mM benzamidin, 1 mM PMSF, 1% aprotinin; (2) 10 mM Tris-HCl pH (7.4), 0.1% Triton X-100, 10% glycerol, 1 mM EDTA, 5 mM benzamidin, 1 mM PMSF, 1% aprotinin; (3) 10 mM lysine-NaOH (pH 9.5), 0.1% Triton X-100, 150 mM NaCl, 10% glycerol, 5 mM benzamidin, 1 mM EDTA; (4) 10 mM Hepes (pH 5.0), 0.1% Triton X-100, 150 mM NaCl, 10% glycerol, 5 mM benzamidin, 1 mM EDTA. The bound EGF-R was eluted from the affinity column by competition for 3 h at 4°C with EGF at a concentration of 1 mg/ml in a buffer containing 10 mM Tris (pH 7.4), 0.05% Triton X-100, 100 mM NaCl, 10% glycerol, 5 mM benzamidin and 1 mM EDTA. After recovery of the receptor the bound EGF was dissociated by changing the pH of the buffer to 10 with NaOH. EGF was completely removed from the receptor preparation by means of ultra filtration on a Centrifugal Ultra Free unit (Millipore, Bedfort, MA, USA). This preparation was used to characterize the purified receptor. For reconstitution experiments the receptor preparations were washed extensively on the same filter unit with the same buffer in which Triton X-100 was replaced by octylglucoside. The purified receptor was stored at −80°C in the same buffer.

SDS-PAGE. Protein determination was done using the BCA-reagent (Pierce, Rockford, IL, USA) with bovine serum albumin (BSA) as standard. Proteins were solubilized in sample buffer (20 mM Tris-HCl (pH 6.8), 33% glycerol, 300 mM dithiothreithol (DTT), 6.7% SDS and 0.01% Bromophenol blue), separated on 10% SDS-PAGE and stained with Coomassie brilliant blue.

Binding of ^{125}I-labelled EGF to the purified receptor. [^{125}I]EGF was prepared by the chloramine-T method [11], specific activity varying between 200000 and 900000 cpm/ng. EGF-binding studies were carried out in a final volume of 200 μl which contained 1 ng of purified EGF-R, 20 mM Hepes (pH 7.4), 0.1% bovine serum albumin (BSA) and 0.1% Triton X-100. [^{125}I]EGF (0.5 ng/ml) and unlabelled EGF were mixed to a final EGF concentration varying from 0.1 (diluted from the stock solution) to 200 ng/ml and binding was allowed for 2 h at room temperature. Non-specific binding of these samples was determined separately by addition of 1000-times excess of unlabelled EGF. Subsequently, the receptor complexes were precipitated by adding 0.5 ml 0.1% γ-globulin in 0.1 M phosphate buffer (pH 7.4) and 0.5 ml of 20.4% poly(ethylene glycol) 6000 at room temperature. The samples were mixed and centrifuged for 5 min at $14000 \times g$. The pellets were washed twice with 8.5% poly(ethylene glycol) in 0.1 M phosphate buffer (pH 7.4). The radioactivity retained in the tubes was measured in a gammacounter (Crystal 5410 Multi Detector Ria system, United Technologies Packard, USA). The binding data were analyzed using the Ligand program [12].

Binding of ^{125}I-labelled EGF to EGF-R-containing vesicles. EGF-R liposomes prepared with 1 μg of receptor and 1 mg lipid were incubated with [^{125}I]EGF (0.5 ng/μl) and unlabelled EGF to final concentrations varying from 0.1 (diluted from stock) to 200 ng/ml in 1 ml of buffer A in the presence of 0.1% BSA. Binding was allowed for 2 h at room temperature. Non-specific binding of the samples was determined separately by addition of a 1000 times excess of unlabelled EGF. Binding to the receptor liposomes was determined by separation of bound and free ligand by filtration (Millipore filtration unit, Millipore) through a glassfiber filter (Whatman, GF-A). The filter was washed twice with 5 ml ice-cold buffer A supplemented with 0.1% BSA. Radioactivity remaining on the filters was determined by counting in a gammacounter as previously described. The binding data were analyzed using the Ligand program [12].

Protein kinase assay of the purified EGF receptor. Autophosphorylation reactions were carried out in a final volume of 50 μl containing 20 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 3 mM $MnCl_2$, 1 mM DTT, The EGF-R (10 ng) was incubated in this buffer with 100 ng/ml EGF for 20 min at 25°C and the kinase reaction was subsequently started by the addition of 10 μM [γ-^{32}P]ATP (10 μCi per assay, Amersham International, UK) and continued for 3 min at 30°C. The reaction was stopped by the addition of sample buffer and the samples were boiled for 3 min. The samples were analyzed on a 10% SDS polyacrylamide gel and autoradiography. The bands of the EGF receptor were excised from the gel and counted with a liquid scintillation counter.

Substrate phosphorylation was measured under the same conditions as described above using 5 mM Angiotensin II as a substrate. The reaction was continued for 3 min at 30°C and stopped by the addition of 5 μl of 50% trichloroacetic acid and 5 μl of bovine serum albumin (10 mg/ml). Proteins were precipitated by centrifugation and 40 μl of the supernatant was spotted onto P81 phosphocellulose filter (Whatman International, Maidstone, UK). The filters were washed four times with 7.5 mM phosphoric acid and counted in a liquid scintillation counter and the results are presented as the mean of three independent experiments.

Protein kinase assay of the reconstituted EGF-R. Receptor (1 μg) containing DOPC vesicles were prepared as described above with the introduction of 4 mM

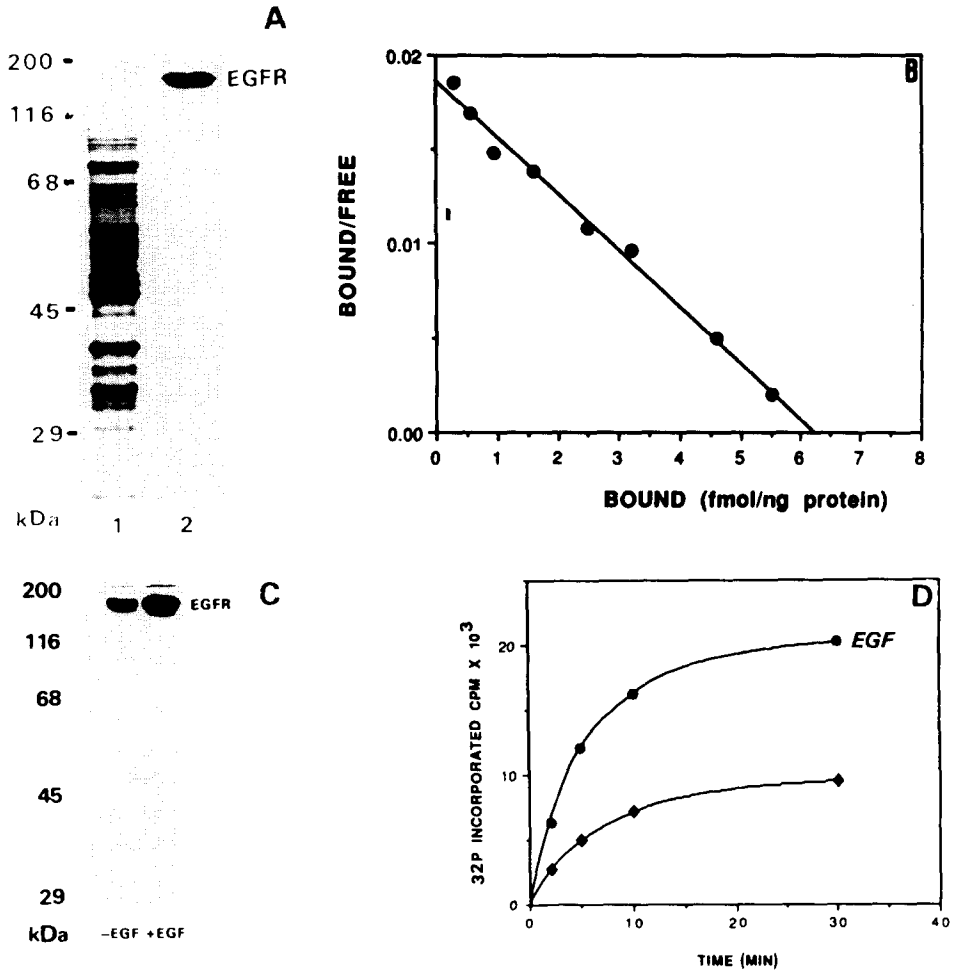

Fig. 1. Purification and characterization of the purified EGF-R. (A) EGF-R was purified from Triton X-100 extracts of A431 cells by affinity chromatography. After eluation the samples were subjected to 10% SDS-PAGE and stained with Coomassie brilliant blue. Lane 1, Triton X-100 extract of A431 cells; lane 2, purified EGF-R. (B) Scatchard analysis of EGF binding to the purified EGF-R. The receptor was incubated with different concentrations of EGF (0.1–200 ng/ml) for 2 h at room temperature. At equilibrium the bound and free EGF were separated by means of centrifugation during 5 min at 14000× g. Non-specific binding was determined by 1000 times excess of non-labelled ligand. Binding data were analyzed using the Ligand program [12]. (C) Autoradiogram of the purified receptor in the absence (1) and presence (2) of EGF. The receptor was first incubated with EGF for 20 min at 25°C and subsequently the kinase reactions were started by the addition of [γ-^{32}P]ATP and continued for 30 min at 30°C. The reactions were stopped by the addition of sample buffer and the samples were analyzed by 10% SDS-PAGE and autoradiography. (D) Time-course of Angiotensin II phosphorylation by the purified EGF receptor in the absence (♦) and presence (●) of EGF. Reactions were carried out in the presence of 5 mM Angiotensin II and 10 ng purified EGF receptor. The reactions were stopped by the addition of 5 μl bovine serum albumin (10 mg/ml) and 5 μl of 50% trichloroacetic acid. After centrifugation, 40 μl of the supernatant was spotted onto P81 phosphocellulose filter, washed four times with phosphoric acid and the filters were counted in a liquid scintillation counter.

Angiotensin II, 5 mM $MgCl_2$ and 10 μM [γ-^{32}P]ATP (10 μCi per assay) during reconstitution. After vesicle formation the non-incorporated receptor and receptor substrate were removed through extensive washing of the vesicles. The vesicles were incubated with 100 ng/ml EGF for 30 min at 20°C. The kinase reaction was stopped and quantitated as described above. Non-specific incorporation of phosphate into Angiotensin II was determined in vesicle preparations without receptor.

Reconstitution of the EGF-R into artificial liposomes. Mixtures of DOPC and cholesterol in different concentrations and mixtures of DOPC with the different phospholipids (2% (w/w) of dioleoylphosphatidylserine (DOPS), dioleoylphosphatidic acid (DOPA), dioleoylphosphatidylinositol (DOPI), dioleoyl-phosphatidylinositol 4-phosphate (DOPIP) and dioleoylphosphatidylinositol 4,5-biphosphate (DOPIP2) in 95% chloroform-5% methanol were dried on the walls of glass tubes, redissolved in ether and redried under vacuum at 20°C for 4 h. The lipid mixtures (1 mg) were dissolved in buffer A (10 mM Tris-HCl (pH 7.4), 100 mM NaCl and 1 mM EDTA) containing 50 mM octylglucoside. The purified EGF-R (1 μg) in 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 10% glycerol, 1 mM EDTA, 5 mM benzamidin, and 50 mM octylglucoside was added and mixed with the different lipid solutions. The solutions were dialysed for 24 h against 3 changes of buffer A. The resulting milky suspension was centrifuged for 1 h at 100 000 $\times g$ to collect the vesicles. The vesicle preparations were washed 3 times to remove non-incorporated receptors and were subsequently subjected to electron microscopy and used in binding studies.

Electron microscopy. For freeze fracture, the receptor liposomes were impregnated with 25% glycerol, frozen in liquid nitrogen and transferred to a Balzers BAF 300 freeze etch machine, fractured and replicated following established procedures [14]. The replicas were stripped off and cleaned with sodium hypochlorite and distilled water as described [14]. The replicas were transferred to grids and examined in a Philips CM10 electron microscope.

Results

Immunoaffinity purification of the EGF-R

The EGF-R was purified from A431 epidermoid carcinoma cells by affinity chromatography using the monoclonal anti EGF-R antibody 528. This antibody recognizes an extracellular peptide determinant located in the EGF-binding domain of the EGF-R [10,15]. Since EGF competes for the similar peptide domain as the antibody 528, EGF has been used to eluate the EGF-R from the affinity column, which appeared to be very effective. EGF was subsequently dissociated from the receptor by changing the pH to 10.0 as has been described before [16] and completely removed from the receptor preparation by means of ultrafiltration. The receptor-containing solution was immediately neutralized. This method of purification resulted in a 76% recovery of the initial receptor population from the Triton X-100 extract, as based on EGF-binding studies and subsequent Scatchard analysis, with a purification factor of 4500 (Table I). The purified receptor fraction was analyzed on 10% SDS-PAGE and after Coomassie brilliant blue staining the gel revealed one single protein band with an apparent molecular weight of 170 kDa (Fig. 1A). Analysis of [^{125}I]EGF binding to the purified receptor, according to the Scatchard method, revealed one single class of binding sites exhibiting an apparent dissociation constant K_d of 2.0 nM (Fig. 1B).

TABLE I

Purification data of the EGF-R

Triton X-100: solubilisate of A431 cells after centrifugation at 100 000 $\times g$ for 1 h. Pure EGF-R: purified EGF-R after elution of the 528 column.

Fraction	Triton X-100	pure EGF-R
Protein (mg)	300	0.05
Activity fmol EGF bound/μg protein	1.2	5500
Total activity fmol EGF bound ($\times 10^3$)	360	275
Recovery (%)	100	76
Purification factor	1	4500

In order to determine the kinase activity of the purified receptor, EGF (100 ng/ml) was added to the protein suspension in the presence of [γ-^{32}P]ATP as described in Materials and Methods. SDS-PAGE, autoradiography and subsequent quantitation (see Materials and Methods) of incorporated radioactivity in the protein band revealed that EGF caused a 2-fold increase in receptor autophosphorylation (Fig. 1C).

Using the peptide Angiotensin II as a substrate for the EGF-R kinase shows that the activation of the receptor by EGF stimulates substrate phosphorylation (Fig. 1D). The K_m for the Angiotensin II substrate is 2.2 mM and the V_{max} is 87 nmol/min per mg in the presence of EGF (data not shown). In conclusion, the EGF-R is purified and can be activated by EGF. This purified receptor was subsequently used in reconstitution experiments.

Reconstitution of the EGF-R into DOPC bilayer vesicles

The purified receptor obtained as described above was dissolved in Triton X-100 which is difficult to remove during reconstitution. Therefore Triton X-100 was exchanged with octylglucoside, which is easily removed by dialysis because of its high critical micelle concentration (25 mM). The receptor preparation was

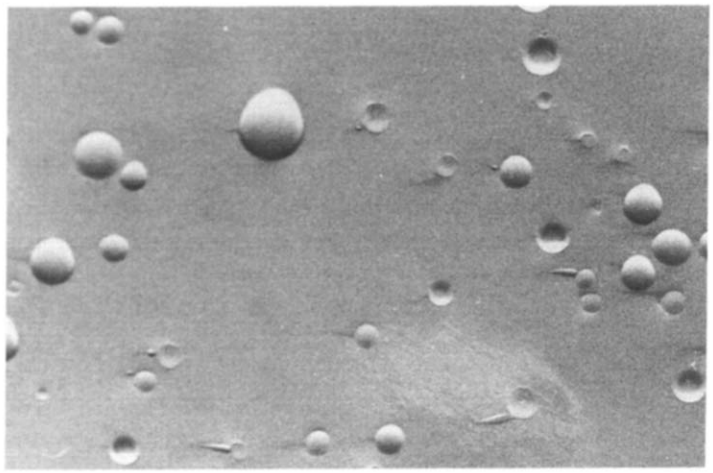

Fig. 2. Freeze-fracture micrograph of EGF-R containing pure DOPC vesicles. Freeze fracture has been performed as described in Materials and Methods. Magnification 100000×.

washed several times with an octylglucoside containing buffer on a filter unit which retained the receptor but not the buffer components.

After displacement of Triton X-100 by octylglucoside the EGF-R-micelles were mixed with DOPC followed by three successive dialysis steps to remove the detergent. The formed liposomes were pelleted by centrifugation at 100000 × g and characterized by electron microscopy, binding studies, receptor sidedness and tyrosine kinase activity for Angiotensin II. Using freeze fracturing it appeared that the pellet contained a rather homogenous population of unilamellar vesicles with a diameter between 400–1500 Å (Fig. 2). No intramembraneous particles (IMP) can be observed in the fracture faces. This is not unexpected since the mass of one receptor is beyond the resolution of the Platium/carbon replica. Also, glycophorin does not show up as an IMP in DOPC reconstitution vesicles [17].

The ability of the reconstituted receptor to bind EGF was tested using [^{125}I]EGF. Scatchard analysis revealed a single class of binding sites exhibiting a K_d of 1.8 nM, which is almost similar to that found for the purified receptor in Triton X-100 micelles (see above). No binding could be detected with vesicles prepared without the receptor. Efficiency of incorporation of the

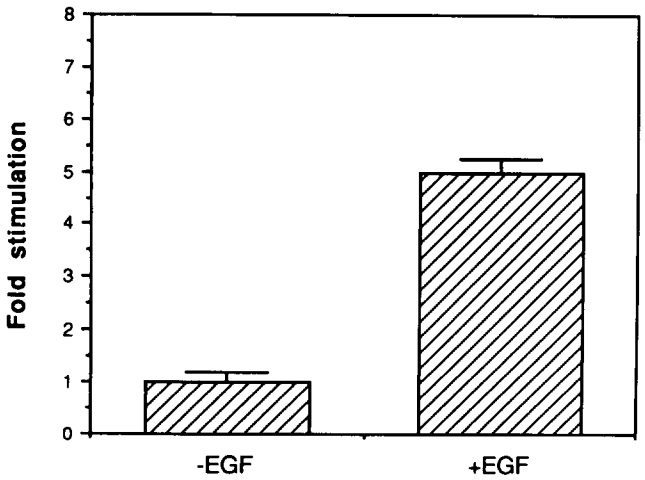

Fig. 3. Angiotensin II phosphorylation by the EGF-R-reconstituted into DOPC vesicles in the absence (−EGF) and presence (+EGF) of EGF. During reconstitution Angiotensin II, Mg^{2+} and ATP were incorporated into the vesicles. After extensive washing the vesicles were incubated with EGF (100 ng/ml) for 30 min at 30°C. Reactions were stopped and quantitated as described in Materials and Methods. Data are represented as fold stimulation of incorporation of phosphate into Angiotensin II. Non-specific phosphate incorporation was determined in vesicle preparations without EGF receptor (bars represent S.E.)

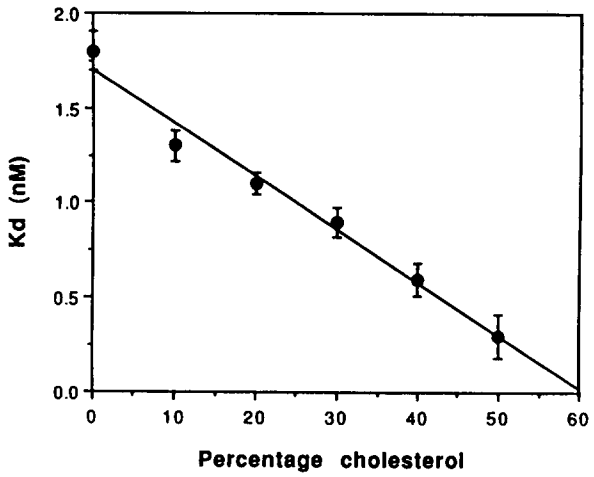

Fig. 4. The effect of cholesterol on EGF-R-binding properties. After reconstitution of the EGF receptor, as described in Materials and Methods, the vesicles were incubated with [^{125}I]EGF (0.5 ng) and unlabelled EGF to final concentrations varying between 0.1–200 ng/ml. Binding was allowed for 2 h at room temperature and binding data were quantitated as described in Materials and Methods. The figure represents the K_d of the EGF-R for EGF as determined by Scatchard analysis in receptor vesicles containing different DOPC/cholesterol ratios. Data are expressed as the average of four independent experiments (bars represent S.E.).

EGF-R into the vesicles was measured by analysis of the number of EGF binding sites associated with the vesicles and appeared to be about 24%. Approx. 76% of the initial added receptor could be recovered from the supernatant after centrifugation of the vesicles.

In order to investigate the sideness of the EGF-R, the vesicles were treated with trypsin. After trypsin treatment no binding of EGF to the vesicles could be detected. Furthermore, analysis with SDS-PAGE revealed that due to the trypsin treatment the EGF-R is cleaved into two peptides with intact vesicles and into three peptides after trypsinization of the solubilized vesicle. These data indicate that the EGF-R has a right-side out transmembrane orientation which is in agreement with results published previously [18].

Subsequently, reconstitution was performed in the presence of Angiotensin II, ATP and Mg^{2+} which enables to investigate substrate phosphorylation of the reconstituted EGF-R. After removing of the non-incorporated EGF-R and substrates, EGF was added to the reconstituted vesicles. A five-fold stimulation in incorporation of ^{32}P was obtained in the Angiotensin II inside the vesicles (Fig. 3). These results demonstrate the functional reconstitution of EGF binding and EGF induced kinase activity, of the EGF-R into DOPC vesicles.

Influence of lipid composition on the EGF-R-binding affinity

First the ability of cholesterol to alter the EGF-binding characteristics of the receptor was investigated, since cholesterol is a normal constituent in eukaryotic plasma membranes and determines overall fluidity. The incorporation of 10 to 50% cholesterol into DOPC vesicles resulted in a gradual increase of the receptor affinity for EGF (Fig. 4).

The affinity of the receptor for EGF changed from 1.8 nM in pure DOPC to 0.3 nM in vesicles with a 1:1 (w/w), DOPC/cholesterol ratio. The reconstitution efficiency of the EGF-R was not altered by the introduction of cholesterol as determined from [^{125}I]EGF binding studies and subsequent Scatchard analysis (data not shown). We also investigated the influence of cholesterol on vesicle size distribution because the diameter of vesicles or cells in suspension could influence the binding aspects of the receptor [19,21]. With increasing amounts of cholesterol incorporated into the liposomes there was no change in the average size of the formed liposomes as seen with freeze fracture (data not shown). So this parameter could not account for the observed modification of the receptor affinity.

Many membrane enzymes have been reported to be dependent on negatively charged phospholipids (for a review, see Ref. 22). In order to establish the influence of lipid composition on receptor affinity, the EGF-R was reconstituted into DOPC vesicles with the addition of low amounts of negativily charged phospholipids. When the EGF-R was reconstituted into liposomes which were composed of DOPC and small amounts (2% (w/w)) of phosphoinositides a 9–18-fold increase in the receptor affinity for EGF was observed (Fig. 5).

The introduction of other charged phospholipids into DOPC vesicles, such as PA and PS, did not lead to an alteration of the affinity of the receptor for EGF (Fig. 5). With the introduction of charged phospholipids the incorporation of the EGF-R into the liposomes was slightly decreased to about 80% in comparison to pure DOPC vesicles, as could be derived from [^{125}I]EGF binding studies and subsequent Scatchard analysis (Fig. 5).

Discussion

Reconstitution studies are a powerful tool to investigate protein-protein and protein-lipid interactions. In this study we investigated the influence of lipids on the

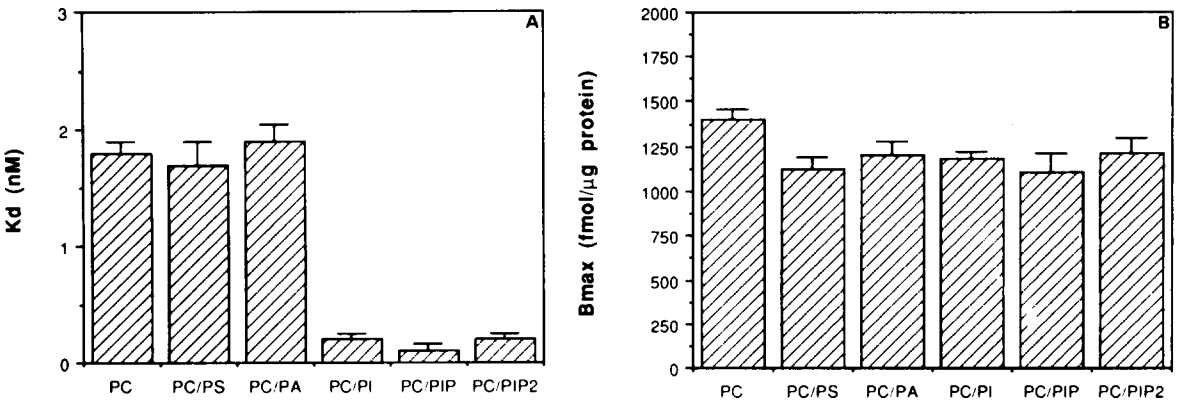

Fig. 5. The effect of different negatively-charged phospholipids on the binding properties of the EGF-R. The K_d and the B_{max} of the reconstituted EGF receptor for EGF determined from Scatchard analysis, as described in Materials and Methods, in vesicles composed of 98% (w/w) DOPC and 2% (w/w) DOPS, DOPA, DOPI, DOPIP and DOPIP2. Data are expressed as the mean of four independent experiments (bars represent S.E.).

EGF-binding properties of the EGF-R. To be able to perform these experiments it was essential to purify a native EGF-R which retained its EGF-binding properties. For this reason we developed a method to isolate the EGF-R with preservation of its EGF-binding capability. In order to measure the level of denaturation of the purified receptor its EGF-binding properties and ability to phosphorylate itself and endogenous substrates was investigated. In the past years several laboratories have reported methods for the purification of the EGF-R. The methods are based on tyrosine-affinity chromatography [23], EGF-affinity chromatography [24] and immunoaffinity chromatography [16,25]. The procedure in this report describes the purification of an EGF receptor, which is still fully EGF-inducible with respect to autophosphorylation and kinase activity. The isolation was done in a single step by means of immunoaffinity chromatography using an antibody directed against the EGF-binding domain, enabling selective eluation of the receptor through competition with EGF. Subsequently, EGF was removed from the purified receptor by changing the pH from 7.4 to 10.0, at which EGF dissociates from the receptor. Separation of EGF and receptor was done by ultra filtration using filters that retained the receptor but not the EGF. The procedure described here yields a highly-purified EGF-R with a recovery of 76%. This is better than the method described in earlier work where the EGF-R was isolated using immunoaffinity chromatography with 2E9, an antibody directed against another extracellular domain of the receptor. The yield with this antibody was approx. 10-times lower than with the antibody used in this study [26]. In short, the method described in this article provides a fast and simple way of obtaining a highly-purified EGF-inducible EGF-R kinase in a single step with high yield. This purified receptor was subsequently used in the reconstitution experiments.

Over the past several years, information has been obtained regarding the lipid effect on binding properties of different membrane receptors [27]. In case of the insulin receptor the affinity of the receptor for insulin increased when the receptor was reconstituted into liposomes composed of saturated lipids [8]. To obtain a fully-functional nicotinic acetylcholine receptor after reconstitution it was essential that besides DOPC and cholesterol also PS and PE were present [28]. So far, only two studies are known on the reconstitution of the EGF-R [18,29]. The first describes a method for reconstitution with no further experiments on its functional properties. In the second study the ability of different GTP-binding proteins to serve as substrates for the EGF-R kinase was examined in reconstituted phospholipid vesicles [29]. In the work presented here, we have used reconstitution approaches to examine the influence of different lipids on the EGF-binding characteristics of the receptor. It was found that the affinity of the receptor for EGF did not change when the receptor was inserted into pure DOPC vesicles as compared to its solubilized form in Triton X-100. The reconstituted receptor retained EGF-inducible kinase activity. When Angiotensin II was used as a receptor kinase substrate, EGF treatment resulted in a five-fold stimulation of phosphate incorporation into this substrate. In conclusion, the EGF-R retained its EGF-binding ability and EGF-induced kinase activity after reconstitution.

Cholesterol is able to change the EGF-binding properties of the EGF-R. The affinity of the receptor for EGF was increased from 1.8 nM in pure DOPC vesicles to 0.3 nM in DOPC vesicles which contained 50% cholesterol. Cholesterol has a condensating effect on the acyl-chain region of fluid lipid bilayers, producing a more ordered, rigid structure [30,31]. In reconstitution studies with the acetylcholine receptor it was found that the presence of cholesterol within the lipid matrix produces a structural stabilization of the receptor protein [32]. The profiles of heat inactivation of this receptor gradually shifted to higher temperatures, as the cholesterol/phospholipid molar ratio in the reconstituted vesicles was increased from 0 to 0.4. The influence of cholesterol on the binding of transferrin to the tranferrin receptor was studied in liposomes [33]. Interestingly, the ratio of cholesterol to phospholipid alters the pattern of transferrin binding to its receptor. The pattern of binding changes from positive cooperativity to non-cooperative as the ratio of cholesterol to phosphatidylcholine increases in the reconstituted vesicles. Until now it is not clear what mechanism underlies the effect of cholesterol on the binding properties of the EGF-R. It was found that the K_d is dependent on receptor density [21], however, we did not observe a change in vesicle size and number of receptors incorporated into vesicles with different cholesterol levels. So the receptor density could not account for the change in receptor binding affinity. It could be possible that cholesterol directly influences the receptor structure as described for the acetylcholine receptor leading to a more stable receptor form. In favour of this hypothesis is the observation that there is a gradual elevation of the receptor affinity towards EGF with increasing amounts of cholesterol incorporated into the vesicles. Another explanation could be that cholesterol influences the rotational and lateral movement of the receptor in the membrane just by altering the membrane fluidity. Further studies are necessary to elucidate at what level cholesterol influences the EGF-binding properties of the EGF-R.

A drastic increase in affinity of the receptor for EGF was found when small amounts of PI lipids were introduced into the DOPC vesicles. The introduction of PS and PA had no effect on the affinity of the

receptor, hence the change in affinity could not be explained by non-specific charge interactions of the lipid headgroups with the receptor. Also, the length of the hydrocarbon chains of the different phospholipids could not account for the observed change in receptor affinity because all the phospholipids used were dioleoyl derivatives. These data suggest that the different phosphoinositide lipids directly influence the conformation of the EGF-R to a more favourable ligand binding form. It is of interest to note that phosphoinositides play an important role in the EGF-induced signal-transduction pathway. One of the first events after EGF binding to the EGF-R is the phosphorylation and activation of PLCγ-1 [34]. Activation of PLC-γ leads to the hydrolysis of a minor membrane phospholipid, phosphatidylinositol 4,5 bisphosphate (PtdIns-4,5-P_2) [2] and the subsequent formation of inositol 1,4,5 trisphosphate (Ins$P$3) and diacylglycerol (DAG). The Ins$P$3 formation induces the release of Ca^{2+} from intracellular stores and DAG is involved in the activation of protein kinase C [5]. Binding of EGF to its receptor stimulates the formation of PtdIns-4,5-P_2 through activation of PtdIns-4-kinase and PtdIns-4P,5-kinase [35,36]. We show that the affinity of the EGF-R can be modulated by the introduction of small amounts of phosphoinositides into DOPC vesicles, and that this effect is not due to aspecific charge interaction. It is therefore tempting to suggest that the interaction of specific phosphoinositides with the EGF-R also plays an important role in the constitution of the receptor high-affinity state. Studies are in progress to investigate whether there is a specific lipid environment around the EGF-R, making use of fluorescent phosphoinositide lipids. The possible association of these phosphoinositides could lead to the situation were not only proteins but also lipids involved in the EGF-R signal-transduction pathway are closely associated with the EGF-R which could lead to a high efficiency of signal transduction.

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