Calmodulin Inhibits the Epidermal Growth Factor Receptor Tyrosine Kinase*

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We demonstrate in this report that the epidermal growth factor (EGF) receptor from rat liver can be isolated by calmodulin affinity chromatography by binding in the presence of Ca²⁺ and elution with a Ca²⁺-chelating agent. The bulk of the EGF receptor is not eluted by NaCl gradient in the presence of Ca²⁺. We ascertained the identity of the isolated receptor by immunoblot and immunoprecipitation using a polyclonal antibody against an EGF receptor from human origin. The purified receptor is autophosphorylated in tyrosine residues in an EGF-stimulated manner, and EGF-dependent phosphorylation of serine residues was also detected. Both the EGF and the transforming growth factor-α stimulate the tyrosine-directed protein kinase activity of the isolated receptor with similar affinities. Furthermore, we demonstrate that calmodulin inhibits the EGF-dependent tyrosine-directed protein kinase activity associated to the receptor in a concentration-dependent manner. This inhibition is partially Ca²⁺ dependent and is not displaced by increasing the concentration of EGF up to an EGF/calmodulin ratio of 10 (mol/mol). In addition, calmodulin was phosphorylated in an EGF-stimulated manner in the presence of a basic protein (histone) as cofactor and in the absence, but not in the presence, of Ca²⁺.

Calcium ion mobilization is an early event during EGF-induced cell proliferation (Moolenaar et al., 1986; Pandiella et al., 1988), and it may be expected that the action of the transient Ca²⁺ rise is directed to multiple targets, since this second messenger controls many cellular functions. However, it is not yet totally understood in detail how the rise in cytoplasmic free calcium ion concentration, directly or indirectly, affects the function of the EGF receptor, particularly its tyrosine kinase activity. Nevertheless, the EGF-induced desensitization of the EGF receptor has been proposed to be mediated by phosphorylation of Thr⁵⁴⁴ by protein kinase C (Whiteley and Glaser, 1986) or phosphorylation of Ser¹⁰⁶⁷ by calmodulin-dependent protein kinase II (Countway et al., 1992).

A great number of calcium-dependent processes are mediated by the intracellular calcium receptor protein calmodulin (Means and Dedman, 1980; Klee et al., 1980; Klee and Vanaman, 1982; Manalan and Klee, 1984). Therefore, we were interested to see whether the EGF receptor could interact with the calcium-calmodulin complex and whether calmodulin was involved in the regulation of its protein-tyrosine kinase activity.

The isolation of the insulin receptor by calmodulin affinity chromatography has been previously reported (Graves et al., 1985) and confirmed by us. Therefore, we thought it was possible that other receptors from the tyrosine-directed protein kinase superfamily could contain a calmodulin-binding domain(s) and consequently be able to interact with, and be modulated by, this regulatory protein.

We have used purified and solubilized plasma membrane fractions from rat liver to carry out our experiments. We report the isolation of the EGF receptor by calmodulin affinity chromatography and demonstrate that calmodulin inhibits the protein-tyrosine kinase activity associated with the receptor. We also show that calmodulin can be phosphorylated in an EGF-stimulated manner by the isolated EGF receptor preparation in the presence, but not in the absence, of histone as cofactor and in the absence, but not in the presence, of calcium ion.

MATERIALS AND METHODS

Chemicals—Radiolabeled [γ-³²P]ATP (triethylammonium salt), (3,000 Ci·mmol⁻¹) (1 Ci = 37 GBq) was purchased from New England Nuclear, and X-Omat AR x-ray films were purchased from Eastman. Molecular weight standards for electrophoresis and PVDF membranes for immunoblot were obtained from Bio-Rad, and bovine brain calmodulin and alkaline phosphatase-conjugated anti-sheep IgG were from Calbiochem. Calmodulin-agarose, ATP (sodium salt), protein A-Sepharose, phosphotyrosine, phosphoserine, phosphothreonine, poly-L-(glutamic acid/tyrosine) (4:1 stoichiometric ratio) (44.5-45.7 kDa), poly-L-lysine, nitroblue tetrazolium, and 5-bromo-4-chloro-3-indoly phosphate were purchased from Sigma. Thin layer (0.1 mm) cellulose-coated chromatographic plates were obtained from Merck, and the polyclonal antibody against the human EGF receptor/c-erbB-2 common epitope from the intracellular domain, residues 929-947 from the c-erb-B2 product (Yamamoto et al., 1986), was purchased from Cambridge Research Biochemicals. Other chemicals used in this work were of analytical grade.

Preparation of Plasma Membrane Fractions—Liver plasma membrane fractions (9-13 mg of protein) were prepared from male Sprague-Dawley albino rats (250-300 g), essentially following the procedure of Brown et al. (1976) as modified by us (San José et al., 1990).

Calmodulin Affinity Chromatography—The solubilization of the...
membranes was performed in 25 mM sodium-Hepes (pH 7.4), 5% (w/v) glycerol, and 1% (w/v) Triton X-100 for 10 min at 4°C. The solubilized membranes were centrifuged at 130,000 g for 1 h, and to the resulting supernatant 100 μM CaCl₂ was added and thereafter passed through a calmodulin-agarose column (4.5-ml bed volume and 4-ml elution volume, equilibrium and wash with 25 mM sodium-Hepes (pH 7.4), 5% (w/v) glycerol, 1% (w/v) Triton X-100, and 100 μM CaCl₂ (Ca²⁺ buffer)). After extensive washing with the Ca²⁺ buffer, the bound proteins were eluted with the same buffer containing 1 mM EDTA instead of CaCl₂, and 0.75-M NaCl fractions were collected. We have found it important to solubilize the membranes in the absence of added Ca²⁺ to prevent partial proteolysis of the receptor by Ca²⁺-dependent proteases (Wang et al., 1989a) possibly present in the membrane fractions. Therefore, CaCl₂ was added immediately before loading the column. We also carried out experiments in which the elution of the receptor by an EGTA buffer containing 150 mM NaCl was preceded by a wash with a NaCl linear gradient (0-150 mM) in the Ca²⁺ buffer. The chromatographic procedures were carried out at 4°C.

Phosphorylation Experiments—Standard phosphorylation experiments, unless indicated otherwise, were carried out at 37°C during 5 min in a total volume of 100 μl in a medium containing: 15 mM sodium-Hepes (pH 7.4), 6 mM MgCl₂, 10 mM [γ-32P]ATP (2-5 Ci/mM), 0.1% (w/v) Triton X-100, 100 μM NaF, 0.2 mM orthovanadate, 10 mM EDTA, and 1% (w/v) Triton X-100. The reaction was initiated upon addition of the radiolabeled ATP and arrested with ice-cold 10% (w/v) trichloroacetic acid. The precipitated material was processed for electrophoresis and autoradiography-Rat liver plasma membranes and the supernatant of the Triton X-100-solubilized membranes, upon labeling with [γ-32P]ATP, electrophoretic separation, and autoradiography, present the phosphorylation pattern shown in the top panel of Fig. 1 (see lanes labeled Membrane and Supernatant, respectively). Most of the phosphorylatable polypeptides do not bind to the calmodulin-agarose in the presence of Ca²⁺ and, therefore, appear in the effluent (see lane labeled Non-bound). Moreover, after extensive washing with the Ca²⁺ buffer the fractions do not show any phosphorylated polypeptides when assayed (see lane labeled Ca²⁺-wash). However, we observe that some protein kinase(s), present in the supernatant of Triton X-100-solubilized plasma membranes, binds to the calmodulin-agarose in the presence of calcium ion, since the latter addition of EGTA to the buffer system results in the elution of a series of polypeptides that are readily autophosphorylated and/or phosphorylated in the presence of [γ-32P]ATP (see lanes labeled EGTA-eluted fractions). The arrow points to a phosphopolypeptide of 170 kDa, identical to the apparent molecular mass of the EGFR receptor. Other phosphopolypeptides of lower molecular mass are also observed in these fractions.

The EGTA-eluted fraction phosphorylates a series of exogenously added protein kinase substrates such as histone, casein, and most importantly, a synthetic copolymer of glutamic acid and tyrosine. The phosphorylation of poly(Glu:Tyr) was strongly stimulated by the presence of EGF (from 2- to 4-fold), and therefore it was of great convenience to follow the protein-tyrosine kinase activity of the putative isolated EGFR receptor (see below). However, the phosphorylation of histone and casein was not significantly stimulated by EGF.

One possibility that should be excluded is that the putative binding of the EGF receptor to the calmodulin-agarose was not mediated by calmodulin but rather by the agarose matrix. This possibility appears to be very unlikely since the elution of the 170-kDa phosphopolypeptide was obtained after chelation of Ca²⁺ from the medium by EGTA. Nevertheless, to ascertain that a nonspecific binding of the putative EGF receptor occurs, we proceeded to perform a linear salt gradient

**RESULTS**

**Isolation of the EGF Receptor by Calmodulin Affinity Chromatography**—Rat liver plasma membranes and the supernatant of the Triton X-100-solubilized membranes, upon labeling with [γ-32P]ATP, electrophoretic separation, and autoradiography, present the phosphorylation pattern shown in the top panel of Fig. 1 (see lanes labeled Membrane and Supernatant, respectively). Most of the phosphorylatable polypeptides do not bind to the calmodulin-agarose in the presence of Ca²⁺ and, therefore, appear in the effluent (see lane labeled Non-bound). Moreover, after extensive washing with the Ca²⁺ buffer the fractions do not show any phosphorylated polypeptides when assayed (see lane labeled Ca²⁺-wash). However, we observe that some protein kinase(s), present in the supernatant of Triton X-100-solubilized plasma membranes, binds to the calmodulin-agarose in the presence of calcium ion, since the latter addition of EGTA to the buffer system results in the elution of a series of polypeptides that are readily autophosphorylated and/or phosphorylated in the presence of [γ-32P]ATP (see lanes labeled EGTA-eluted fractions). The arrow points to a phosphopolypeptide of 170 kDa, identical to the apparent molecular mass of the EGFR receptor. Other phosphopolypeptides of lower molecular mass are also observed in these fractions.

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Calmodulin Inhibits the EGF Receptor Kinase

Fig. 1. Isolation of the EGF receptor by calmodulin affinity chromatography. Top panel, plasma membranes (78 μg of protein) (lane labeled Membrane), the supernatant of the Triton X-100-solubilized membranes (60 μl) (lane labeled Supernatant), the non-bound material to the calmodulin-agarose column in the presence of Ca²⁺ (60 μl) (lane labeled Non-bound), the material eluted after an extensive wash with the Ca²⁺ buffer (60 μl) (lane labeled Ca²⁺-wash), or the indicated EGTA-eluted fractions (80 μl) (lanes labeled by the fraction number) were assayed for 5 min at 37°C in 100 μl of a medium containing 20 mM sodium-Hepes (pH 7.4), 6 mM MgCl₂, 0.6% (w/v) Triton X-100 (except in the membrane assay where 0.1% was used), 3% (w/v) glycerol (except in the membrane assay where was absent), and 10 μM [γ⁻³²P]ATP (4 μCi). The reaction was arrested upon addition of 10% (w/v) ice-cold trichloroacetic acid and the precipitated proteins processed for electrophoresis and autoradiographs as described under “Materials and Methods.” A representative autoradiograph is presented. The arrow points to the 170-kDa EGF receptor. Bottom panel, plasma membranes were solubilized as described under “Materials and Methods.” The Triton X-100 supernatant was passed through the calmodulin-agarose column and eluted first with a linear NaCl gradient (0–150 mM) in the presence of Ca²⁺, and thereafter with EGTA, in the presence of 150 mM NaCl, as described under “Materials and Methods.” The plot presents the EGF-dependent phosphorylation of poly(Glu:Tyr) by the different eluted fractions (filled symbols). The assays of aliquots of the different fractions (50 μl) were carried out in a total volume of 100 μl at 37°C for 5 min in 15 mM sodium-Hepes (pH 7.4), 6 mM MgCl₂, 1 mM EGTA, 50 μM CaCl₂ (free Ca²⁺ 4.5 mM), 100 μg/ml poly(Glu:Tyr), 0.5% (w/v) Triton X-100, and 10 μM [γ⁻³²P]ATP (2 μCi), in the absence and presence of 1 μM EGF. The reaction was arrested with 10% (w/v) trichloroacetic acid, the precipitated acids processed for electrophoresis and autoradiographs, and the intensity of °P-labeling of poly(Gly:Tyr) was measured as described under “Materials and Methods.” The activity in the absence of EGF was subtracted from the activity in its presence. The conductivity of the eluted fractions was measured with a potenciometer (open symbols).

elution with NaCl from 0 to 150 mM in the presence of Ca²⁺, previous to the elution with EGTA. The bottom panel of Fig. 1 presents a plot of the EGF-dependent protein-tyrosine kinase activity (phosphorylation of poly(Glu:Tyr) in the presence of EGF minus phosphorylation of poly(Glu:Tyr) in the absence of EGF) eluted from the calmodulin-agarose column, first by the NaCl gradient and subsequently upon addition of EGTA. It is clearly shown in this figure that the majority of the EGF-dependent phosphorylation of poly(Glu:Tyr) takes place in the fractions eluted with EGTA, with only a lower EGF-dependent protein-tyrosine kinase activity present in the fractions eluted with high concentrations of NaCl in the presence of calcium ion. It is important, however, to emphasize that the EGF-dependent protein-tyrosine kinase activity eluted with NaCl is zero just before the addition of EGTA. This experiment further supports our view that the retention of the putative EGF receptor in the calmodulin-agarose column is mediated by calmodulin.

To perform a semiquantitative estimation of the amount of the phosphorylatable 170-kDa polypeptide that is retained by the calmodulin-agarose column in the presence of Ca²⁺ (see Fig. 2), we assayed, in the presence of EGF, the Triton X-100 supernatant and the material that does not bind to the calmodulin-agarose column in the presence of Ca²⁺. Thereafter, identical amounts of protein from the Triton X-100 supernatant before loading the column (lane 1) and the material that does not bind to the column in the presence of calcium ion (lane 2) were processed for electrophoresis and autoradiographs. It can be observed that the only °P-labeled band...
that drastically decreases in the column effluent is the 170-kDa phosphopolypeptide. This phosphopolypeptide is recovered after elution with EGTA (lane 3). Notice that for practical reasons the amount of EGTA-eluted fraction assayed and processed for electrophoresis (lane 3) was only a small fraction of the total obtained material.

To ascertain that the EGTA-eluted 170-kDa phosphopolypeptide was indeed the EGF receptor, we performed immunological experiments testing the recognition of the 170-kDa phosphopolypeptide by a polyclonal antibody against an EGF peptide. As can be observed in Fig. 4 (right panel), the level of phosphorylation of the EGF receptor was dependent on the concentration of the ligand. In Fig. 4 (right panel), a plot of the level of phosphorylation of the receptor at different concentrations of EGF is presented. An apparent $K_{d}$ of approximately $3.10^{-7}$ M for EGF in the phosphorylation process was calculated from this plot.

Nevertheless, the coincidence in molecular mass of the 170-kDa phosphopolypeptide and the signal detected in the immunoblot was not the only criteria we followed to demonstrate that the EGF receptor was indeed isolated from the calmodulin-agarose column. In Fig. 3 (right panel), our results of the immunoprecipitation experiments are presented. In lane 1 the autoradiographs of the EGTA-eluted fraction assayed in the absence (–) and presence (+) of EGF, and in lane 2 the immunoprecipitated 170-kDa phosphopolypeptide assayed in the absence (–) and presence (+) of EGF are presented. Notice the overexposure of these autoradiographs and that other phosphopolypeptides of lower molecular mass, also present in the EGTA-eluted fraction, were not detected in the immunoprecipitate.

These experiments definitively demonstrated that the 170-kDa phosphopolypeptide isolated from the calmodulin-agarose column was indeed the EGF receptor.

The Phosphorylation of the Isolated EGF Receptor Is Stimulated by EGF and TGFα—Fig. 4 (left panel) shows that the phosphorylation of the isolated EGF receptor was stimulated not only by EGF (compare lanes 1 and 2) but by TGFα as well (compare lanes 1 and 3). Furthermore, the phosphorylation of the EGF receptor was dependent on the concentration of the ligand. In Fig. 4 (right panel), a plot of the level of phosphorylation of the receptor at different concentrations of EGF is presented. An apparent $K_{d}$ of approximately $3.10^{-7}$ M for EGF in the phosphorylation process was calculated from this plot.

As can be observed in Fig. 4 (right panel), the level of phosphorylation of the EGF receptor obtained by saturating concentration of the ligand was only slightly above 2-fold with respect to the phosphorylation in the absence of the ligand. Therefore, it was of interest to determine the nature of the phosphorylated amino acids in the receptor isolated from the calmodulin-agarose column, both in the absence and in the presence of EGF, to see whether the basal phosphorylation in

![Fig. 3. Immunoblot and immunoprecipitation of the isolated EGF receptor. Left panel, Triton X-100 supernatant from solubilized plasma membrane (160 mg of protein) (lanes 1, 3, and 5) was assayed at 37 °C for 5 min in 100 ml of a medium containing 15 mM sodium-Hepes (pH 7.4), 6 mM MgCl$_2$, 0.1% (w/v) Triton X-100, 1 mM EGF, and 10 μM [γ-32P]ATP (2 μCi). The peak EGTA-eluted fraction (140 ml) (lanes 2, 4, and 6) was assayed at 37 °C for 5 min in 200 ml of a medium containing 17.5 mM sodium-Hepes (pH 7.4), 6 mM MgCl$_2$, 0.7 mM EGTA, 0.7% (w/v) Triton X-100, 3.5% (w/v) glycerol, 1 mM EGF, and 10 μM [γ-32P]ATP (2 μCi). The reaction was arrested with 10% (w/v) trichloroacetic acid and the precipitated proteins processed for electrophoresis. Thereafter, the proteins were electrotransferred to a PVDF membrane and processed for immunoblots and autoradiographs as described under "Materials and Methods." Lanes 1 and 2 present the immunoblots with the anti-EGF receptor antibody developed with the anti-sheep IgG antibody conjugated with alkaline phosphatase. Lanes 3 and 4 present the control for the immunoblots in the absence of the anti EGF receptor antibody. Lanes 5 and 6 present the autoradiographs of the dry PVDF membranes. The arrows point to the 170-kDa EGF receptor. Right panel, the peak EGTA-eluted fraction (50 ml) was incubated at 37 °C for 5 min in a total volume of 100 ml of a medium containing 15 mM sodium-Hepes (pH 7.4), 6 mM MgCl$_2$, 0.5 mM EGTA, 0.5% (w/v) Triton X-100, 2.5% (w/v) glycerol, and 10 μM [γ-32P]ATP (4 μCi), in the absence (–) and presence (+) of 1 mM EGF. The reaction was arrested upon addition of 170 μl of 125 mM EDTA, 50 mM NaF, and 1% (w/v) Triton X-100 and the mixture subjected to immunoprecipitation as described under "Materials and Methods." Representative autoradiographs of the EGTA-eluted fraction (lanes 1, ± EGF) and the immunoprecipitated material (lanes 2, ± EGF) are presented. The arrow points to the 170-kDa EGF receptor.
was assayed at different concentrations of EGF. The isolated EGF receptor with 10% (w/v) trichloroacetic acid, and the precipitated proteins under "Materials and Methods." Representative autoradiographs are shown. The arrow points to the 170-kDa EGF receptor. Right panel, the assays were performed as in the left panel but in the presence of the indicated concentration of EGF. The plot presents the average intensities of labeling of the EGF receptor in the autoradiographs at different concentrations of EGF. The error bars represent the standard error of six different experiments.

the absence of EGF was due to phosphorylation of other amino acid instead of tyrosine. However, the phosphoaminoacid analysis of the 170-kDa phosphoplypeptide, assayed in the absence of EGF, demonstrated the presence of phosphotyrosine only. In contrast, when we assayed the receptor in the presence of EGF an increase in the levels of phosphotyrosine and the appearance of phosphoserine was observed as well (see Fig. 5).

The low apparent affinity of the isolated receptor for EGF, as measured by the phosphorylation of the receptor itself (see Fig. 4, right panel), was confirmed measuring its protein-tyrosine kinase activity toward poly(Glu:Tyr) as shown in Fig. 6. The levels of phosphorylation of the exogenous substrate were dependent on the concentration of EGF (filled symbols) or TGFα (open symbols), and we calculated an identical apparent K_0.5 of approximately 3.10^{-7} M for both ligands, when phosphorylation of poly(Glu:Tyr) was used as a measurable parameter. The protein-tyrosine kinase activity of the EGTA-eluted fraction toward exogenous substrate was very high in the absence of any ligand, and only increases, in this particular experiment, slightly above 2.5-fold at saturating concentrations of EGF or TGFα (see Fig. 6).

Calmodulin Inhibits the Protein-Tyrosine Kinase Activity of the Isolated EGF Receptor—Our next step was to test the effect of calmodulin on the protein-tyrosine kinase activity of the EGF receptor isolated from the calmodulin-agarose column. In Fig. 7, a plot of the inhibitory effect of calmodulin
on the protein-tyrosine kinase activity of the EGF receptor is presented. The inhibitory action of calmodulin was somewhat more pronounced in the presence (filled symbols) than in the absence (open symbols) of EGF. We calculated from this plot an apparent \( K_i \) for calmodulin of approximately 1 \( \mu \text{M} \) when the total protein-tyrosine kinase was considered and of approximately 0.2-0.3 \( \mu \text{M} \) when only the EGF-dependent activity was considered. We have also demonstrated that the EGTA-eluted fraction phosphorylates histone and casein, and that calmodulin inhibits (apparent \( K_i \) for calmodulin of approximately 2-6 \( \mu \text{M} \)) the phosphorylation of both protein substrates. Nevertheless, we did not observe a significant EGF-stimulated phosphorylation of these substrates (results not shown).

We have to exclude the presence of a calmodulin-dependent phosphoprotein phosphatase (calcineurin) activity in the EGTA-eluted fraction isolated from the calmodulin-agarose column. Table I demonstrates the absence of any significant calmodulin-dependent phosphatase activity in the plasma membrane fractions used in this study. The tests were performed not only in the presence of Ca\(^{2+}\) but in the presence of Mn\(^{2+}\) and Ni\(^{2+}\), two metals that are well known to stimulate the activity of calcineurin (Wang et al., 1989b). We have to emphasize that the use of p-nitrophenylphosphate has been shown by us to be an optimal substrate to assay the calmodulin-dependent phosphatase activity in the EGTA-eluted fraction isolated from the calmodulin-agarose column. The number in parenthesis indicates the number of preparations.

For this reason, we have also tested the effect of increasing the EGF/calmodulin ratio (mol/mol) on the protein-tyrosine kinase activity, when the inhibitory action of calmodulin was assayed. Fig. 8 shows that by increasing the EGF/calmodulin ratio up to 10 (mol/mol) the inhibitory action of calmodulin on the phosphorylation of poly(Glu:Tyr) is not significantly prevented.

We have also studied the requirements of different concentrations of free Ca\(^{2+}\) for the inhibitory effect of calmodulin on the protein-tyrosine kinase of the isolated EGF receptor using an EGTA-Ca\(^{2+}\) buffer system. We have found that Ca\(^{2+}\) is not an absolute requirement for the inhibitory action of calmodulin. Therefore, some inhibition (approximately 50%) was observed in the presence of EGTA alone (results not shown).

![Fig. 8. EGF does not prevent the inhibitory action of calmodulin on the protein-tyrosine kinase activity of the isolated EGF receptor.](image)

**Table I**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Total phosphatase activity (nmol·min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>None</td>
<td>252 ± 92 (2)</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>214 ± 91 (2)</td>
</tr>
<tr>
<td>Ca(^{2+}) + CaM</td>
<td>206 ± 136 (2)</td>
</tr>
<tr>
<td>Ca(^{2+}) + Mn(^{2+})</td>
<td>197 ± 108 (2)</td>
</tr>
<tr>
<td>Ca(^{2+}) + Mn(^{2+}) + CaM</td>
<td>202 ± 118 (2)</td>
</tr>
<tr>
<td>Ca(^{2+}) + Ni(^{2+})</td>
<td>286 (1)</td>
</tr>
<tr>
<td>Ca(^{2+}) + Ni(^{2+}) + CaM</td>
<td>286 (1)</td>
</tr>
</tbody>
</table>

Therefore, the use of p-nitrophenylphosphate has been shown by us to be an optimal substrate to assay the calmodulin-dependent phosphatase activity of calcineurin (Wang et al., 1989b). Table I shows that the level of phosphatase activity in the EGTA-eluted fraction was drastically reduced, and again any significant calmodulin-dependent phosphatase activity was not detected. Moreover, the data in Table I represent the total phosphatase activity present in the EGTA-eluted fraction (approximately 4 ml). However, our assays on phosphorylation of poly(Glu:Tyr), where we study the inhibitory effect of calmodulin, contain only from 30 to 50 \( \mu \text{l} \) of this fraction. Therefore, the final phosphatase activity (that as mentioned previously is not calmodulin-dependent) present in the calmodulin inhibition assays can be considered negligible (from 0.02 to 0.06 nmol·min\(^{-1}\)).

Furthermore, we have also tested the effect of increasing the EGF/calmodulin ratio (mol/mol) on the protein-tyrosine kinase activity, when the inhibitory action of calmodulin was assayed. Fig. 8 shows that by increasing the EGF/calmodulin ratio up to 10 (mol/mol) the inhibitory action of calmodulin on the phosphorylation of poly(Glu:Tyr) is not significantly prevented.

We have also studied the requirements of different concentrations of free Ca\(^{2+}\) for the inhibitory effect of calmodulin on the protein-tyrosine kinase of the isolated EGF receptor using an EGTA-Ca\(^{2+}\) buffer system. We have found that Ca\(^{2+}\) is not an absolute requirement for the inhibitory action of calmodulin. Therefore, some inhibition (approximately 50%) was observed in the presence of EGTA alone (results not shown).

However, the inhibition of the protein-tyrosine kinase activity induced by calmodulin was greater at progressively higher concentrations of free Ca\(^{2+}\) in the assay system (see Fig. 9). The enhancement by Ca\(^{2+}\) of the inhibitory action of calmodulin (open symbols) was observed both in the presence and in the absence of EGF (compare circles and triangles in Fig. 9).

**Calmudulin Is Phosphorylated in an EGF-stimulated Manner**—We have also demonstrated that the EGTA-eluted fraction isolated from the calmodulin-agarose column phosphorylates calmodulin and that this process was stimulated by EGF. The phosphorylation of calmodulin requires the presence of a basic protein such as histone, as can be observed in Fig. 10 (panels A and B). We have also demonstrated the occurrence of phosphorylation of calmodulin using poly(Lys) as a cofactor, although in this case we did not observe a significant stimulatory effect of EGF (results not shown). In panel A, it is observed that in the absence of histone we did not detect any phosphorylation of calmodulin (compare lanes 1 and 2 with lanes 3 and 4). The absence of phosphorylation of calmodulin was observed in the absence (lane 3) and presence (lane 4) of EGF. However, in the presence of histone calmodulin is phosphorylated very efficiently in an EGF-stimulated manner (see lanes 7, 8, 11, and 12). Notice that EGF was present in the experiments shown in lanes 8 and 12. Histone is phosphorylated as well during the assay (see lanes 5, 6, 9, and 10 in the absence of calmodulin, and lanes 7, 8, 11, and 12 in the presence of calmodulin). Therefore, in order to better observe the phosphorylated form of calmodulin we added EGTA to the electrophoresis sample buffer in order to induce a calcium-dependent electrophoretic mobility shift of phosphocalmodulin. The presence of EGTA in the electro-
was assayed at 37 °C for 5 min in the absence (filled symbols) and in the presence (open symbols) of 1 μM calmodulin. The reaction was arrested with 10% (w/v) trichloroacetic acid and the precipitated proteins processed for electrophoresis and autoradiographs. The 32P-labeled poly(Glu:Tyr) was measured as described under "Materials and Methods." The plot represents the levels of phosphorylation of poly(Glu:Tyr) versus the concentration of free calcium ion.

**Fig. 9.** Effect of the concentration of free Ca²⁺ on the inhibitory action of calmodulin on the protein-tyrosine kinase activity of the isolated EGF receptor. EGTA-eluted fraction (90 μl) was assayed at 37 °C for 5 min in 100 μl of a medium containing 15 mM sodium-Hepes (pH 7.4), 6 mM MgCl₂, 0.5 mM EGTA, 0.5% (w/v) Triton X-100, 1.5% (w/v) glycerol, 100 μg/ml poly(Glu:Tyr), 10 μM [γ-32P]ATP (2 μCi), 0.3 mM EGTA, and different concentrations of CaCl₂ to yield the indicated concentrations of free calcium ion, in the absence (triangles) and in the presence (circles) of 1 μM EGFP, and in the absence (filled symbols) and in the presence (open symbols) of 1 μM calmodulin. The reaction was arrested with 10% (w/v) trichloroacetic acid, and the precipitated proteins processed for electrophoresis and autoradiographs. The 32P-labeled poly(Gly:Tyr) was measured as described under "Materials and Methods." The plot represents the levels of phosphorylation of poly(Glu:Tyr) versus the concentration of free calcium ion.

**Fig. 10.** EGF-stimulated phosphorylation of calmodulin by the isolated EGF receptor preparation. Panel A, EGTA-eluted fraction (50 μl) was assayed at 37 °C for 5 min in 100 μl of a medium containing 15 mM sodium-Hepes (pH 7.4), 6 mM MgCl₂, 0.5 mM EGTA, 0.5% (w/v) Triton X-100, 2.5% (w/v) glycerol, 100 μg/ml histone, 1 μM EGFP (lanes 2, 4, 6, 8, 10, and 12), 10 μM CaCl₂ (histone (lanes 5, 7, 8, 9, 11, and 12), and 10 μM [γ-32P]ATP (2 μCi). The reaction was arrested with 10% (w/v) trichloroacetic acid and the precipitated proteins were processed for electrophoresis and autoradiographs as described under "Materials and Methods." The experiment showing that most of the EGF receptor is retained in the calmodulin-agarose column in the absence of added EGTA to the electrophoresis sample buffer (lanes 1-8) to induce an electrophoretic mobility shift of phosphocalmodulin to 21 kDa. In the absence of added EGTA to the electrophoresis sample buffer, phosphocalmodulin migrates as a 21-kDa polypeptide (see arrows). Panel B, EGTA-eluted fraction (50 μl) was assayed at 37 °C for 5 min in 100 μl of a medium containing 15 mM sodium-Hepes (pH 7.4), 6 mM MgCl₂, 0.5 mM EGTA, 0.5% (w/v) Triton X-100, 2.5% (w/v) glycerol, 100 μg/ml histone, 1 μM EGFP (lanes 2 and 4), 1 μM calmodulin (lanes 3 and 4), and 10 μM [γ-32P]ATP (2 μCi), in the absence (lanes 1 and 2) and presence (lanes 3 and 4) of 0.6 mM CaCl₂ (100 μM free Ca²⁺). The reaction was arrested with 10% (w/v) trichloroacetic acid and the precipitated proteins processed for electrophoresis and autoradiographs as described under "Materials and Methods," except that 10 mM EGTA was added to the electrophoresis sample buffer (lanes 1-4) to induce an electrophoretic mobility shift of phosphocalmodulin to 21 kDa (see arrow).
Calmodulin Inhibits the EGF Receptor Kinase

affect the binding of target calmodulin-binding proteins to calmodulin due to the drastic increase in ionic strength, and therefore we used a more moderate concentration of salt to perform these experiments. The elution of a small part of the EGF receptor protein-tyrosine kinase activity with approximately 100 mM NaCl in the presence of Ca²⁺ is not surprising. It could represent the activity corresponding to the elution of free monomers resulting from the dissociation of EGF receptor dimers bound to calmodulin by only one of the monomers that could remain bound to calmodulin while the other is eluted.

The involvement of the calmodulin-dependent protein kinase II in the phosphorylation of serine residues in the EGF receptor, and the subsequent inhibition of its protein-tyrosine kinase (Countaway et al., 1992), could suggest that we have copurified this calmodulin-dependent protein kinase by calmodulin affinity chromatography, and therefore, it could explain the observed effect of calmodulin on the protein-tyrosine kinase activity of the receptor. Although this remains a remote possibility, we wish to emphasize that in liver this protein kinase is localized in the cytoplasmic domain of the human EGF receptor, and the subsequent inhibition of its protein-tyrosine kinase activity of the receptor. This effect could be mediated by the direct interaction of calmodulin with the EGF receptor. Hence, protein kinase C has been shown to be modulated by calmodulin (Zhao et al., 1992) and is unlikely to be present in great extent in the plasma membrane preparations used by us. Furthermore, we have not observed any significant stimulation of the phosphorylation of histone or casein by calmodulin, when the EGTA-eluted fraction was used as source of protein kinase. On the contrary, calmodulin induces a pronounced inhibition on the phosphorylation of these substrates.

Countaway et al. (1992) indicate in their work that the purified EGF receptor was a poor substrate for calmodulin-dependent protein kinase II possibly due to the use of detergents in the EGF receptor isolation procedure. We wish to point out that we solubilize the EGF receptor with 1% (w/v) Triton X-100 and that our assays of inhibition of the protein-tyrosine kinase by calmodulin were performed in the presence of 0.3–0.5% (w/v) of the same detergent. Thus, the possibility that the inhibitory effect of calmodulin could be mediated by this protein kinase is more difficult. Nevertheless, we have detected EGF-dependent phosphorylation of serine residues in the isolated EGF receptor. Consequently, future work should be conducted to analyze the origin and role of this phosphorylation in the isolated receptor. The inhibitory action of calmodulin on other protein kinases has been noticed. Hence, protein kinase C has been shown to be modulated by calmodulin (Zhao et al., 1991). Therefore, we do not exclude the possibility that phosphorylation of the EGF receptor at other sites besides tyrosine residues could be central in the inhibitory effect of calmodulin observed by us.

We have not detected, however, the presence of a calmodulin-dependent phosphoprotein phosphatase in our EGF receptor preparations (see Table I). Furthermore, the inhibitory action of calmodulin was observed in the presence of 25 μM vanadate (results not shown), an inhibitor that has been proven to be effective at this concentration to efficiently prevent the dephosphorylation of the EGF and the insulin receptors (King and Sale, 1988). Therefore, it remains possible that the action of calmodulin on the protein-tyrosine kinase activity could be mediated by the direct interaction of calmodulin with the EGF receptor.

Analysis of calmodulin-binding domains in different proteins has not shown a common consensus sequence in all the proteins. However, the alternative clustering of basic amino acids and hydrophobic amino acids capable of forming amphipathic helices has been recognized as an important feature in calmodulin-binding domains. We have noticed that the region of the cytoplasmic domain of the human EGF receptor, adjacent to the transmembrane domain (amino acids 645–659), where Thr⁶⁵⁴ is located, is very rich in basic amino acids and non-polar amino acids (Arg-Arg-Arg-His-Ile-Val-Arg-Lys-Arg-Thr-Leu-Arg-Arg-Leu-Leu) (Ullrich et al., 1984), and therefore has the characteristics of a calmodulin-binding site. Further work should be carried out to determine if this region is indeed a calmodulin-binding site. Nevertheless, the possibility did not escape to our attention that the binding of calmodulin to this region could result in a conformational change similar to the one probably occurring during the phosphorylation of Thr⁶⁴⁴, and hence inactivating the protein-tyrosine kinase activity of the receptor in a similar fashion.

We wish to emphasize that two different mechanisms of interaction of calmodulin with the EGF receptor appear to exist. On one the hand the EGF receptor binds to the calmodulin–agarose column in a calcium-dependent manner. However, the inhibitory action of calmodulin on the protein-tyrosine kinase activity of the receptor is only partially dependent on calcium ion. Furthermore, the phosphorylation of the EGF receptor by the EGF receptor only occurs in the absence of calcium ion. This suggests the existence of more than one calmodulin-binding site in the EGF receptor.

The interaction of calmodulin with their target proteins usually requires Ca²⁺ (Means and Dedman, 1980; Klee et al., 1980; Klee and Vanaman, 1982; Manalan and Klee, 1984). However, Ca²⁺-independent interactions of calmodulin with some target proteins also play a prominent role in cellular processes (Cohen et al., 1978; Anderson and Gopalakrishna, 1985). Therefore, it is not surprising that the two modes of interaction can be observed in the EGF receptor.

The phosphorylation of calmodulin by the insulin receptor, in the presence of basic proteins or synthetic polycations, has been well documented (Graves et al., 1986; Sacks and McDonald, 1988; Sacks et al., 1989). Consequently, it is interesting to observe that the phosphorylation of calmodulin by the EGF receptor presents similar characteristics of phosphorylation of this protein by the insulin receptor. These observations perhaps underscore a common basic mechanism in the phosphorylation of calmodulin by receptors of the protein-tyrosine kinase superfamily.

Calmodulin has long been implicated in the regulation of cell proliferation. Therefore, the demonstration that one of the points of regulation is located at the level of entry of one of the mitogenic signals (EGF receptor) adds further interest to the study of the mechanism of action of calmodulin in mitosis.

REFERENCES
