Regulation of the Ligand-Dependent Activation of the Epidermal Growth Factor Receptor by Calmodulin*

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1The abbreviations used are: BAPTA-AM, 1,2-bis-(o-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid tetra(acetoxymethyl) ester; CaM, calmodulin; CaM-BD, CaM-binding domain; CaMK-II, CaM-dependent protein kinase II; DMEM, Dulbecco’s modified Eagle’s medium; ECL, enhanced chemiluminescence; EGF, epidermal growth factor; EGFR, EGF receptor; FACS, fluorescence-activated cell scanning; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GF109203X, 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide; HRGβ1, heregulin-β1; KN-93, 2-[(N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide); LDH, lactate dehydrogenase; mCtrl-p, myristoylated control myosin light-chain kinase mutant peptide; mLCK-p, myristoylated myosin light-chain kinase peptide; PKC, protein kinase C; PLCγ1, phospholipase Cγ1; PVDF, poly(vinylidene difluoride); SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; W-7, N-(6-aminoxyethyl)-5-chloro-1-naphthalenesulfonamide; W-13, N-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide.
Background: The EGFR binds calmodulin (CaM).

Result: CaM antagonists, CaM down-regulation in conditional CaM-KO cells, chelation of Ca$^{2+}$ and mutagenesis of the CaM-binding domain inhibit EGFR activation.

Conclusion: The Ca$^{2+}$/CaM complex is a positive regulator of the EGFR.

Significance: This is the first work with a multi-approach strategy demonstrating that CaM directly regulates the EGFR in living cells.

Calmodulin (CaM) is the major component of calcium signaling pathways mediating the action of various effectors. Transient increases in the intracellular calcium level triggered by a variety of stimuli lead to the formation of Ca$^{2+}$/CaM complexes, which interact with and activate target proteins. In the present study the role of Ca$^{2+}$/CaM in the regulation of the ligand-dependent activation of the epidermal growth factor receptor (EGFR) has been examined in living cells. We show that addition of different cell permeable CaM antagonists to cultured cells or loading cells with a Ca$^{2+}$ chelator inhibited ligand-dependent EGFR autophosphorylation. This occurred also in the presence of inhibitors of protein kinase C, CaM-dependent protein kinase II and calcineurin, which are known Ca$^{2+}$- and/or Ca$^{2+}$/CaM-dependent EGFR regulators, pointing to a direct effect of Ca$^{2+}$/CaM on the receptor. Furthermore, we demonstrate that down-regulation of CaM in conditional CaM knock out cells stably transfected with the human EGFR decreased its ligand-dependent phosphorylation. Substitution of six basic amino acid residues within the CaM-binding domain (CaM-BD) of the EGFR by alanine resulted in a decreased phosphorylation of the receptor and of its downstream substrate phospholipase C$\gamma$1. These results support the hypothesis that Ca$^{2+}$/CaM regulates the EGFR activity by directly interacting with the CaM-BD of the receptor located at its cytosolic juxtamembrane region.

The epidermal growth factor as well as many other cell stimulatory factors induces an early and transient increase in the cytosolic concentration of free Ca$^{2+}$ (for review see 1-3). This brief Ca$^{2+}$ mobilization event orchestrates different strategies to inactivate the intrinsic tyrosine kinase activity of the EGFR1 after delivery of the mitogenic signal (for reviews see 4, 5). Among these regulatory mechanisms are those leading to the phosphorylation of the receptor by PKC and CaMK-II at distinct threonine and serine residues (6, 7).

Our laboratory has shown that the Ca$^{2+}$/CaM complex binds to the EGFR in vitro and in vivo and regulates its activity in cultured cells (8-13). Previous work has demonstrated that the CaM binding domain (CaM-BD) of the receptor is located at its cytosolic juxtamembrane region (10, 13, 14-17), and appears to be responsible for the observed inhibition of the tyrosine kinase activity of the receptor in vitro (8, 9). However, more recently experimental evidences suggest that in living cells Ca$^{2+}$/CaM could play an activating role (12, 13, 15). Different mechanistic models have been proposed to account for this stimulatory action of the Ca$^{2+}$/CaM complex (reviewed in 5): i) by releasing the positively charged CaM-BD from the negatively charged inner leaflet of the plasma membrane, as this electrostatic interaction will otherwise maintain the receptor in an auto-inhibited state in the absence of ligand (13, 15, 16); ii) by releasing the positively charged CaM-BD from a negatively charged sequence denoted the CaM-like domain (CaM-LD) located C-terminally of the tyrosine kinase domain, an interaction that could also contribute to stabilize the EGFR dimer after ligand binding (18-20).

Activation of the EGFR upon ligand-induced dimerization appears to occur by an asymmetric allosteric mechanism where the C-terminal lobe of the kinase domain of one of the monomers interacts with the N-terminal lobe of the apposed monomer, thus forming an active dimer (21). The intracellular juxtamembrane region of the receptor, which contains the CaM-BD, has been shown to be indispensable for this allostery activation mechanism to be operative (22-24), further giving credential to the possible implication of CaM in the activation process. Nevertheless, the actual mechanism by which CaM plays this activating role is not yet known.

In this report we present new evidence demonstrating that the Ca$^{2+}$/CaM complex plays a positive role in the ligand-dependent activation of the EGFR in cultured cells using CaM antagonists as well as conditional CaM-KO cells. Replacement of six out of eight positive charged residues within the CaM-BD of the receptor by alanine dramatically impairs its activating capacity, suggesting that the direct interaction of Ca$^{2+}$/CaM with the EGFR at the juxtamembrane region is responsible for this regulation.

**EXPERIMENTAL PROCEDURES**

Reagents - Fetal bovine and chicken sera, DMEM, RPMI-1640 media and the ATP determination kit were obtained from Invitrogen. The ECL kit was purchased from GE Healthcare, and the X-ray films were from GE Healthcare (Hyperfilm™,MP) or Eastman Kodak (X-Omat AR). A23187 (free acid, from Streptomyces chartreusensis), BAPTA-AM, W-7, W-13, GF109203X, KN-93,
cyclosporine A (from Trichoderma polysporum), monoclonal (mouse IgG<sub>2a</sub>) anti-EGFR antibody (clone 528) recognizing the extracellular region, and the protease inhibitor cocktail were purchased from Calbiochem. The pre-stained molecular mass standards for electrophoresis were from Bio-Rad. Fast Green FCF, tetracycline, ionomycin (free acid, from Streptomyces conglutatus), and anti-tubulin-α monoclonal (mouse) antibody (clone DM 1A) were purchased from Sigma. Mouse EGF (from submaxillary glands) was obtained from Sigma or Upstate Biotechnology, and human recombinant EGF was from PeproTech EC. Monoclonal (mouse IgG<sub>1</sub>) anti-CaM antibody; and monoclonal (mouse IgG<sub>2a</sub>) anti-phosphotyrosine antibody (clone 4G10) were obtained from Upstate Biotechnology. The monoclonal (rabbit IgG) anti-EGFR antibody (clone E235) recognizing the C-terminal region, monoclonal (mouse IgG<sub>1</sub>) anti-phospho-EGFR (Tyr1045) antibody (clone 11C2), and the monoclonal (mouse IgG<sub>1</sub>) anti-CaM antibody were obtained from Millipore. Polyclonal (rabbit) anti-phospho-PLCγ<sub>1</sub> (Tyr1253) and anti-EGFR (1005) antibodies, monoclonal (mouse) anti-PLCγ<sub>1</sub> antibody (clone E-12), and secondary anti-mouse IgG coupled to horseradish peroxidase were purchased from Santa Cruz Biotechnology. Polyclonal (rabbit) anti-GAPDH antibody coupled to horseradish peroxidase (RC20H) was obtained from Transduction Laboratory. Blasticidin S, EGF-tetramethylrhodamine, goat anti-mouse IgG (H + L) antibody conjugated to Alexa Fluor<sup>488</sup>, and goat anti-rabbit IgG (H + L) antibody conjugated to horseradish peroxidase were obtained from Invitrogen. The myristoylated peptide corresponding to the CaM-BD of MLCK and the control low-affinity MLCK mutant peptide (25) were a kind gift of Dr. Katalin Török from the St. Georges Hospital Medical School, London UK. Other chemicals used in this work were of analytical grade.

**Cells cultures** - The cell lines used in this study were: EGFR-T17 and N7xHERe murine fibroblasts stably transfected with the human EGFR but with distinct expression levels (see supplementary Fig. S1); N7x and Swiss 3T3 murine fibroblasts expressing negligible and low levels of endogenous EGFR, respectively; human lung adenocarcinoma A549 cells, a cell line expressing a moderate level of endogenous EGFR; human epidermoid carcinoma A431 cells overexpressing the EGFR; human neuroblastoma NB69 cells expressing moderate levels of endogenous EGFR; human embryonic kidney HEK293 cells devoid of endogenous expression of EGFR; human breast adenocarcinoma SK-BR-3 cells overexpressing ErbB2 and a much lower levels of EGFR and ErbB3 (26); DT40wt/EGFR cells, a chicken pre-B lymphoma cell line (27) stably transfected with the human EGFR; and the ET1-55/EGFR conditional CaM-KO cell line stably transfected with the human EGFR, where the expression of recombinant rat CaM is negatively controlled by a tetracycline response element (Tet-Off system) (Panin et al., manuscript in preparation). The ET1-55/EGFR cell line was originally derived from DT40 CaMIL<sup>−/−</sup> cells (28). All adherent cell lines were propagated in DMEM supplemented with 2 mM L-glutamine, 40 µg/ml gentamicin and 10 % (v/v) FBS, except for NB69 cells where 15 % (v/v) FBS was used, in a humidified atmosphere of 5 % (v/v) CO<sub>2</sub>; in air at 37 ºC. The DT40wt/EGFR and ET1-55/EGFR cells were grown in suspension in RPMI-1640 medium containing 10 % (v/v) fetal bovine serum, 1 % (v/v) chicken serum, 2 mM L-glutamine, and 40 µg/ml gentamicin as above but at 40 ºC. The cells were maintained for 24 h in the absence of serum before performing the experiments. When CaM downregulation in ET1-55/EGFR cells was required, treatment with 1 µg/ml tetracycline was performed for the time indicated in the legend to the figures.

**Expression vectors and cell transfection** - The human wild type EGFR (with the sequence 645RRRHIVRKTLLRLQ<sup>660</sup> at its CaM-BD) and a mutant receptor with the modified sequence 645AAHIVAATLLRLQ<sup>660</sup> that was denoted as EGFRmA1A2 (and in abbreviated form A1A2) both cloned in the pcDNA6A vector (Invitrogen) as previously described (29) were kindly provided by Dr. Mien-Chie Hung from the University of Texas MD Anderson Cancer Center, Houston TX. The HEK293 cells were transiently transfected with the wild type or the A1A2 mutant EGFR as follows; cells were seeded in P6 multi-well plates (7.5 × 10<sup>4</sup> cells per well) and grown in medium plus 10 % (v/v) FBS as indicated above to approximately 80 % confluence. The cells were treated with 25 µM chloroquine for 15 min, and thereafter transfected with 5 µg of vector DNA (empty pcDNA6A, pcDNA6A/EGFRwt or pcDNA6A/EGFRmA1A2) in 1 ml of a buffer containing 124 mM CaCl<sub>2</sub>, 25 mM Hepes-NaOH (pH 7.05), 5 mM KCl, 125 mM NaCl, 0.75 mM Na<sub>2</sub>HPO<sub>4</sub> and 6 mM glucose. After 8-10 h of incubation the medium was replaced and the cells were maintained for 24 h in DMEM with 10 % (v/v) FBS. The medium was then replaced by serum-free DMEM 24 hours before performing the experiments. Wild type DT40 cells, and ET1-50 and ET1-55 cells were stably transfected with the pcDNA6A/EGFR wt vector by electroporation, and the clones were selected by treatment with 10 µg/ml blasticidin S for two weeks.

**Cell viability** – The number of viable cells was measured by the Trypan blue exclusion method as described (30). As a surrogate marker of cell disruption the amount of LDH activity in the culture medium was monitored as described (31).

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*References cited in the text are available in the original manuscript.*
In addition, the content of intracellular ATP was followed using a luciferase assay kit (Invitrogen Molecular Probes™) essentially as described (32). This also allowed ascertaining that no limitation was imposed on EGFR phosphorylation upon stimulation of the cells with EGF.

EGFR activation assay in living cells – Adherent cells were grown to confluence in P6 multi-well plates as indicated above and maintained in 1.5 ml of FBS-free medium for 24 h before performing the experiments. The non-adherent DT40wt/EGFR and ET1-55/EGFR cells were grown in suspension and serum-starved for 4 h before performing the experiments. When CaM down-regulation was required, the ET1-55/EGFR and DT40 wt/EGFR cells (negative control) were treated with 1 µg/ml tetracycline for the time indicated in the figure legends prior to EGFR stimulation. In the experiments involving inhibitors of PKC (GF109203X), CaMK-II (KN-93) and/or calcineurin (cyclosporine A); CaM antagonists (W-7, W-13 or mMLCK peptide); the Ca²⁺ ionophore A23187; and the Ca²⁺ chelator BAPTA-AM, the cells were pre-treated with the compounds prior to stimulation with 10 nM EGF. The reaction was stopped upon removal of the medium and addition of 1 ml of ice-cold 10 % (w/v) trichloroacetic acid. Cells were collected in Eppendorf tubes and centrifuged at 16,000 g for 10 min. The supernatant was discarded and the cells were lysed with Laemmli buffer and processed for SDS-PAGE and Western blot analysis as described below. The TCA method allows the quick termination of the phosphorylation reaction and to more efficiently prevent the spurious dephosphorylation of EGFR than the classical technique describe in (33) using a RIPA buffer containing 50 mM Tris-HCl (pH 8), 1 % (w/v) sodium deoxycholate, 0.1 % (w/v) sodium dodecyl sulfate, 1 % (w/v) p-nitrophenyl-phosphate, 1 % (w/v) sodium fluoride, 1 mM sodium orthovanadate, 1 mM PMSF, and 5 µl/ml of protease inhibitor cocktail. Thus, the TCA method may be advantageous for determining EGFR auto(trans)phosphorylation in living cells (supplementary Fig. S2).

Electrophoresis and Western blots - Proteins were separated by slab SDS-PAGE in 5-20 % (w/v) linear gradient of polyacrylamide and 0.1 % (w/v) sodium dodecyl sulfate at pH 8.3. When required, 5-10 mM EGTA was added to the sample buffer to attain a mobility of CaM to 21 kDa (34). After processing by SDS-PAGE, proteins were electrotransferred from the gel to a PVDF membrane (or a nitrocellulose membrane in the case of CaM) for 2 h at 300 mA in a buffer containing 48 mM Tris-base, 36.6 mM l-glucose, 0.04 % (w/v) SDS and 20 % (v/v) methanol; fixed with 0.2 % (v/v) glutaraldehyde in 25 mM Tris-HCl (pH 8), 150 mM NaCl and 2.7 mM KCl (TNK buffer) for 45 min; and transiently stained with Fast Green to ascertain the regularity of the transfer procedure. The membranes were blocked with 5 % (w/v) bovine serum albumin or 5 % (w/v) fat-free powdered milk, as recommended by the antibody suppliers, in 0.1 % (w/v) Tween-20, 100 mM Tris-HCl (pH 8.8), 500 mM NaCl and 0.25 mM KCl (T-TBS buffer). This was followed by probing overnight at 4 °C using a 1/2000 dilution of the corresponding primary antibody, and thereafter for 1 h at room temperature using a 1/5000 dilution of the appropriate secondary anti-IgG antibody coupled to horseradish peroxidase. The bands were visualized upon development with the ECL reagents following instructions from the manufacturer and exposure of X-ray films for appropriate periods of time. The intensities of the bands were quantified with a computer-assisted scanning densitometer using the NIH Image 1.60 program.

Detection of the EGFR at the cell surface - Cells were detached with 2 mM EDTA avoiding trypsin treatment, washed with phosphate buffer saline containing 0.1 % (w/v) bovine serum albumin and incubated with 4 µg/ml EGF-tetramethylrhodamine for 30 min at 4 °C in the dark and analysis by flow cytometry using the FL2-H (585 nm) channel. Control cells were either kept untreated to detect background fluorescence, or pre-incubated with 10 µM non-labeled EGF to prevent EGF-tetramethylrhodamine binding to measure non-specific binding. Alternatively, EGFR was labeled using an anti-EGFR antibody recognizing the extracellular region of the receptor and an Alexa Fluor®488-labeled anti-mouse IgG secondary antibody and the signal was analysed using the FL1-H (488 nm) channel. The following controls were included: i) an anti-EGFR antibody recognizing the intracellular region of the receptor to ascertain cell intactness; ii) absence of primary antibodies to measure non-specific binding; and iii) absence of any antibody to measure the background fluorescence. The low fluorescence attained in all these controls was subtracted from the mean signal attained with the antibody recognizing the EGFR extracellular region.

Other analytical procedures - Protein concentration was determined by the BCA method (35) after precipitation with 10 % (w/v) trichloroacetic acid, and using bovine serum albumin as a standard. Where appropriate the results are presented as the mean ± SEM of the densitometry of the bands detected by Western blot after loading correction using protein stained by Fast Green, GAPDH or tubulin-α; or the EGFR expressed at the cell surface as determined by FACS. Otherwise representative data from separated experiments repeated 2-5 times with essentially identical results are presented.

RESULTS
Cell-Permeable CaM Antagonists Inhibit EGF-Dependent EGFR Activation – We first showed that cells treated with a variety of cell-permeable CaM antagonists exhibit a lower EGF-dependent auto(trans)phosphorylation of the receptor. The naphthalenesulfonamide derivative W-7 inhibits the EGF-dependent activation of the receptor in EGFR-T17 fibroblasts, albeit with very low efficiency but in a dose-dependent manner (Figs. 1A-B). In contrast, a stronger inhibition was obtained using a myristoylated peptide corresponding to the CaM-BD of the enzyme MLCK (mMLCK-p) that specifically sequesters intracellular CaM with high affinity (25). The treatment with a control myristoylated MLCK peptide (mCtrl-p) with very low affinity for CaM due to the substitution of a critical tryptophan by tyrosine (25), has little inhibitory capacity. Similar results were obtained when the activation of the ErbB2 receptor by HRGβ1 was tested in human breast adenocarcinoma SK-BR-3 cells (see supplementary Fig. S3). ErbB2 is a member of the ErbB tyrosine kinase family closely related to the EGFR. ErbB2 also binds CaM and may be regulated by this modulator (36, 37).

We tested the effect of the CaM antagonist W-13 on the EGF-dependent activation of EGFR in N7xHERc fibroblasts, which express a lower level of the receptor than EGFR-T17 fibroblasts (supplementary Fig. S1). To avoid possible interference from known Ca2+- and Ca2+/CaM-dependent kinases regulating EGFR, such as PKC and CaMK-II (6, 7), or the Ca2+/CaM-dependent phosphatase calcineurin (38), the cells were pre-treated with inhibitors for PKC (GF109203X), CaMK-II (KN-93) and calcineurin (cyclosporine A). W-13 efficiently inhibited the initial spike of the EGF-dependent activation of EGFR in the absence (Figs. 1C and 1E), and most efficiently in the presence (Figs. 1D and 1F) of the Ca2+ ionophore A23187 that triggers a massive increase of intracellular calcium. The enhanced inhibition attained by W-13 in the presence of A23187 was also observed in the presence of ionomycin as an alternative Ca2+ ionophore (data not shown). These results were essentially the same as those previously reported from experiments done in the same cell line in the absence of cyclosporine A (13), suggesting that calcineurin is not involved in the process. We have previously demonstrated in N7xHERc fibroblasts that the low affinity analogue W-12 was far less efficient in inhibiting the EGF-dependent phosphorylation of the EGFR as compared to W-13 and W-7 (12).

EGFR-T17 and N7xHER murine fibroblasts expressed high and low levels of the EGFR, respectively, as compared to a panel of other cell lines analyzed for total EGFR levels by Western blotting (supplementary Figs. S1A and S1B) and cell surface expression by FACS (supplementary Figs. S1C and S1D). Thus, the inhibitory effect of CaM antagonists on EGFR activation can be observed in cells with distinct receptor expression levels.

Buffering of Intracellular Ca2+ Decreases EGF-Dependent EGFR Activation – To determine whether the presumed activating effect of CaM on EGFR in living cells required the presence of Ca2+, we buffered intracellular Ca2+ by loading NB69 and A549 cells with BAPTA-AM as these cells were best suited for Ca2+ buffering experiments. The time-course of EGF-dependent activation of the EGFR was lower in NB69 cells when loaded with 50 µM BAPTA-AM, particularly in its initial phase (2-5 min) (Fig. 2A). This inhibitory effect was more apparent at higher concentrations of BAPTA-AM, not only in NB69 cells but also in A549 cells as well (Fig. 2B). Quantitative determination showed an apparent IC50 ≈ 100 µM for BAPTA-AM in NB69 cells (Fig. 2C). Our results are in agreement with earlier reports showing that BAPTA-AM inhibited EGFR phosphorylation in other cellular systems (39, 40).

CaM Downregulation in Conditional CaM-KO Cells Inhibits EGF-Dependent EGFR Activation – To further ascertain that CaM is implicated in EGF-dependent EGFR auto(trans)phosphorylation we stably transfected ET1-55 conditional CaM-KO cells (Panina et al., manuscript in preparation) with the human EGFR. The expression levels of CaM in the conditional CaM-KO cells (clones ET1-50 and ET1-55) are far higher than in the wild type DT40 cells (Panina et al., manuscript in preparation). Addition of tetracycline efficiently down-regulated CaM expression in the ET1-55/EGFR cells, while no effect was observed in the control DT40/wt/EGFR cells (Fig. 3A). As CaM is vital for the cell and the levels of CaM expression are drastically reduced after 48-72 hours of exposure to tetracycline, we tested several parameters of cell viability. We could demonstrate that the number of living cells, the extent of cell lysis, and the level of intracellular ATP were similar in control DT40/wt/EGFR cells (Fig. 3B) and ET1-55/EGFR cells (Fig. 3C) up to 72 hours with tetracycline, indicating that the number of living cells, the extent of cell lysis, and the level of intracellular ATP were similar in control DT40/wt/EGFR cells (Fig. 3B) and ET1-55/EGFR cells (Fig. 3C) up to 72 hours with tetracycline, even when CaM expression was drastically reduced in the latter (see Fig. 3A). These results are consistent with additional tests performed in non-transfected CaM-KO ET1-50 cells, where cell mortality only appears to significantly increase after 4-5 days of exposure to tetracycline (Panina et al., manuscript in preparation).

We found that the EGFR activity measured by assaying its auto(trans)phosphorylation was dependent on the level of CaM (Fig. 3D). In addition, we compared the time-courses of EGF-dependent EGFR activation in the absence of tetracycline and after different times of exposure to the antibiotic, both in DT40/wt/EGFR cells (Fig. 3D).
the Ca2+/CaM complex plays a significant role in In this report, we present evidence showing that also reported to exert some regulatory functions. modified (e.g. phosphorylated) CaM, (45), was addition, apo-CaM (42, 44) and post-translationaly and modulate a multitude of targets (43). In EGFR in living cells. We show that not only phosphorylation of its downstream substrate the A1A2 mutant EGFR, we tested the kinase downstream activity was also impaired in (Fig. 4B). To investigate whether the tyrosine mutant EGFR denoted A1A2. Six out of the eight basic amino acid residues located in its CaM-BD were changed to alanine to prevent CaM binding. The mutant EGFR was clearly less phosphorylated than the wild type receptor upon EGF stimulation (Fig. 4B). To investigate whether the tyrosine kinase downstream activity was also impaired in the A1A2 mutant EGFR, we tested the phosphorylation of its downstream substrate PLCγ1. The EGF-dependent phosphorylation of PLCγ1 was much lower in cells transfected with the mutant (A1A2) than with the wild type (wt) EGFR (Fig. 4C). As expected, no PLCγ1 phosphorylation signal was detected in the mock-transfected cells using the empty vector (EV). Equal efficiency of the transfection procedure was confirmed, as the expression of total EGFR (Fig. 4D) and the EGFR at the cell surface (Fig. 4E) are shown to be identical for the wild type (wt) and the mutant (A1A2) receptors. The absence of endogenous EGFR in the mock-transfected cells (EV) was further confirmed (Fig. 4D).


different and very selective cell permeable CaM antagonists inhibit the ligand-dependent activation of the receptor, but CaM downregulation in conditional CaM-KO cells stably transfected with the human EGFR also results in similar lower activation of the receptor upon EGF addition. In previous reports we demonstrated that CaM antagonists exert an inhibitory action on the EGFR when Ca2+ and Ca2+/CaM-dependent regulatory systems acting on the receptor were operative (12) or inhibited, particularly PKC and CaMK-II, (13). Calcineurin, a Ca2+/CaM-dependent phosphatase, has been shown to dephosphorylate the EGFR (38). This phosphatase not only acts on phospho-Ser/Thr but also on phospho-Tyr esters, albeit with lower efficiency (46, 47). Thus, it was not clear whether calcineurin could activate or inhibit some unidentified regulatory system(s) acting on the EGFR. Therefore, in this report we tested the effect of the CaM antagonist W-13 after suppressing not only PKC and CaMK-II as previously reported (13), but inhibiting calcineurin as well. Our results demonstrate that even in this triple-inhibited system, W-13 decreases the EGF-dependent activation of the EGFR, not only in the absence of A23187, but with greater efficiency in the presence of the ionophore. This greater inhibitory efficiency was expected, as the concentration of the target of W-13, the Ca2+/CaM complex, is higher when intracellular Ca2+ is increased upon treatment with the ionophore.

Myristoylated peptides capable of penetrating the plasma membrane and entering the intracellular space proved to be a useful tool in cell biology research (48). In the present study, we used a highly specific CaM-binding myristoylated peptide, mMLCK-p, corresponding to the CaM-BD of MLCK (25) to sequester intracellular CaM. Treatment of cells with mMLCK-p resulted in the decrease of the ligand-induced activation of both, the EGFR and the ErbB2 receptor, further supporting the regulatory role of CaM.

The differential inhibitory effect of W-7, W-13, mMLCK-p and mCtrl-p on the EGF-dependent phosphorylation of the EGFR in the different cell lines tested may represents not only their differential affinity for CaM as a major factor, but also subtle differences in their membrane permeability.

We also demonstrated that buffering of the cytosolic free Ca2+ with BAPTA-AM also decreased the EGF-dependent activation of the EGFR in NB69 and A549 cells. Although loading cells with 100 µM BAPTA-AM has been shown to decrease intracellular ATP levels to half of its normal concentration (49), this should pose little threat for an efficient phosphorylation of the EGFR, at least in the neuroblastoma NB69 cells, as the cytosolic concentration of ATP in basal
conditions in neural cells ranges from 0.6 ± 0.1 to 1.5 ± 0.4 mM depending of the cell type (50), and the affinity constant of ATP for the EGFR is lower than 10 µM (51, 52).

The experiments performed with the tetracycline-dependent CaM-KO cells strongly support the notion that CaM is involved in the ligand-dependent activation of the EGFR. Tetracycline exerts multiple changes in the gene transcriptional program of mammalian cells, affecting for instance endocytosis and other membrane related processes (53). To compensate for possible effects of tetracycline on EGFR internalization we corrected the phosphorylation signal with the amount of receptor present at the cell surface, instead of using the total EGFR signal as determined by Western blot. Our results indicate that CaM downregulation in ET1-55/EGFR cells indeed inhibits EGFR-dependent EGFR auto(trans)phosphorylation. This in combination with the calcium buffering experiments discussed above supports the notion that it is the Ca²⁺/CaM complex, and not apo-CaM, the species that plays a stimulatory role on EGFR activation.

We have previously identified a Ca²⁺-dependent CaM-BD in the cytosolic juxtamembrane region of the EGFR (5, 10). Here we show that CaM appears to activate the EGFR by its direct interaction with the CaM-BD in living cells, since the mutant EGFR (A1A2), expected to be incapable of CaM binding, has a lower capacity to be activated by EGF and hence is less efficient in phosphorylating its downstream substrate PLCγ1 as compared to the wild type EGFR. This is consistent with our earlier results showing that the inhibitory action of the CaM antagonist W-13 was absent in a mutant EGFR harboring the insertion of a non-relevant acidic amino acid sequence splitting the CaM-BD in two halves and supposedly unable to bind CaM (12, 13). Moreover, our results are also in agreement with previous reports showing that deletion of the cytosolic juxtamembrane region of the EGFR results in an inactive receptor (19, 54, 55; Jellali and Villalobo, unpublished results). In addition, the mutation of each individual amino acid residue of the cytosolic juxtamembrane region by alanine-scanning mutagenesis negatively affects the ligand-mediated activation of the EGFR (23), which underscores the relevance of the CaM-BD for this process. This argues against a rather unspecific effect relying on the altered charge of this segment in the EGFR. Nevertheless, we cannot exclude that these mutations could also affect the receptor in other aspects unrelated to CaM binding.

A model by which Ca²⁺/CaM could activate the EGFR in living cells proposes that this complex releases the positively charged CaM-BD of the receptor bound to the negatively charged inner leaflet of the plasma membrane upon activation of the receptor by EGF, hence preventing the autoinhibitory action that this electrostatic interaction may otherwise exert in the absence of the ligand (13, 15, 16). This model supports the view of the asymmetric allosteric activation mechanism proposed by the group of Kuriyan (21), as the CaM-BD appears to be essential for EGFR dimerization (19, 22). Moreover, crystallographic studies of the EGFR have shown that its CaM-BD is indeed in close association to the inner leaflet of the plasma membrane (24).

We have previously demonstrated that W-13 is able to stimulate EGFR phosphorylation in the absence of ligand, possibly due to its amphipathic weak alkaline nature, as it might disrupt the electrostatic interaction of the CaM-BD of the receptor with the inner leaflet of the plasma membrane and hence releasing the autoinhibition (13). Therefore, it is possible that the Ca²⁺/CaM complex, formed upon exposing cells to agents able to increase the cytosolic concentration of Ca²⁺, could exert similar effect.

How could the clear inhibitory action that the Ca²⁺/CaM complex has on the EGFR in vitro be explained in the light of the observed activation induced by this complex in living cells? A possible answer could be that no membrane is present in the detergent-solubilized receptor, and the Ca²⁺/CaM complex may bind to the EGFR at its exposed CaM-BD mimicking the inhibitory electrostatic interaction of this segment with the inner leaflet of the plasma membrane. In living cells, however, the Ca²⁺/CaM complex could actively pull out the CaM-BD from the inner leaflet of the membrane thus relieving the autoinhibition exerted by this interaction (16). This is further supported by the fact that the detergent-solubilized EGFR is partially active (≈ 50 %) in the absence of EGF, as compared to the receptor in its native environment where its activity in the absence of EGF is absent or very low (8, 10).

Overall, our results demonstrate that the Ca²⁺/CaM complex exerts a positive regulatory role on the EGFR, thus connecting the Ca²⁺ signal generated by this receptor with its functionality. This opens the possibility that the CaM-dependent regulatory mechanism exerted on the EGFR, as well as the ErbB2, could lead to a better understanding of oncogenic ErbB receptors signaling.

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REFERENCES

LEGENDS TO THE FIGURES

FIGURE 1. CaM antagonists decrease EGF-dependent EGFR activation in living cells. (A) Serum-starved EGF-R-T17 fibroblasts were incubated for 15 min with increasing concentrations of the CaM antagonists W-7 (13, 26 and 40 µM) and mMLCK-p (5, 10 and 15 µM), and the low affinity control peptide mCtrl-p (5, 10 and 15 µM) as indicated. Controls in the absence (-) of CaM antagonists are also shown. EGF (10 nM) was added where indicated and the reaction arrested with ice-cold 10 % (w/v) trichloroacetic acid 2 min thereafter. The phosphorylated form of the EGFR was detected using an anti-phosphotyrosine antibody. (B) The plot presents the mean ± range (n = 2) EGF-dependent EGFR phosphorylation versus the concentration of W-7 (triangles), mMLCK-p (circles), and mCtrl-p (squares) from experiments identical to the one shown in panel A. Error bars are shown if larger than the symbols. (C, D) Serum-starved N7xHERc fibroblasts were incubated for 2 h at 37 ºC with 10 µM KN-93 (CaMK-II inhibitor), 10 µM GF109203X (PKC inhibitor) and 1 µg/ml cyclosporine A (calcineurin inhibitor). Thereafter, the cells were incubated for 15 min in the absence and presence of 40 µM W-13 as indicated, followed by an additional incubation for 15 min in the absence (C) and presence (D) of 5 µg/ml A23187. Finally, 10 nM EGF was added and the reaction was arrested as above 1, 2, 5, 10, 15, 30 or 60 min after EGF addition. Controls in the absence (-) of EGF are also shown. The phosphorylated EGFR band was detected using an anti-phosphotyrosine antibody. (E, F) The plots present the mean ± SD of three (E) or two (F) independent experiments identical to the ones shown in panels C and D, in the absence (E) and presence (F) of A23187, and in the absence (open symbols) and presence (filled symbols) of W-13, relative to controls performed in the absence of inhibitors and ionophore and treated with 10 nM EGF for 1 min (100 %). Error bars are shown if larger than the symbols.

FIGURE 2. Loading cells with BAPTA-AM decreases EGF-dependent EGFR activation. (A) Serum-starved NB69 cells were incubated in the absence (Ctrl) and presence of 50 µM BAPTA-AM (BAPTA-AM) for 30 min at 37 ºC. The cells were washed and treated with 10 nM EGF for the indicated times and the reaction arrested with ice-cold 10 % (w/v) trichloroacetic at the indicated time after EGF addition. Controls in the absence (-) of EGF are also included. The phosphorylated form of the EGFR and tubulin-α, as a loading control, were detected using anti-phosphotyrosine and anti-tubulin-α antibodies. (B) The experiment was performed as described in panel A except that NB69 and A549 cells were loaded with progressive concentrations of BAPTA-AM (20, 50, 100, 300 and 500 µM) and treated with 10 nM EGF for 2 min. Controls in the absence (-) of BAPTA-AM are also presented. The phosphorylated EGFR was detected using an anti-phosphotyrosine antibody (in NB69 cells) or an anti-phospho(Tyr1045)-EGFR antibody (in A549 cells). Segments of the PVDF membrane stained with Fast Green or using an anti-GAPDH antibody are presented as loading controls. (C) The plot presents the mean ± range (n = 2) of independent experiments performed with NB69 cells, relative to a control in the absence of BAPTA-AM (100 %). Error bars are shown if larger than the symbols.

FIGURE 3. CaM down-regulation in conditional CaM-KO cells regulates EGF-dependent EGFR activation. (A) DT40wt/EGFR and ET1-55/EGFR cells were incubated with 1 µg/ml tetracycline for the indicated time. The expression of CaM was determined using an anti-CaM antibody. (B, C) The plots present the mean ± SEM (n = 3) cell viability (circles), lactic dehydrogenase released to the medium (triangles) and the amount of intracellular ATP (squares) determined in DT40wt/EGFR (B) and ET1-55/EGFR (C) cells after treatment with 1 µg/ml tetracycline for the indicated time. Error bars are shown if larger than the symbols. (D) Serum starved ET1-55/EGFR cells were incubated in the absence and presence of 1 µg/ml tetracycline as indicated. The cells were incubated with 10 nM EGF and the reaction was arrested with ice-cold 10 % (w/v) trichloroacetic acid at the indicated times after EGF addition. The phosphorylated form of the EGFR was determined using an anti-phosphotyrosine antibody, total EGFR using an anti-EGFR antibody, and CaM using an anti-CaM antibody. Loading controls using anti-tubulin-α and anti-GAPDH antibodies are also shown. (E, F) The plots present the mean ± SEM (n = 5) EGFR phosphorylation (indistinctly of the absence or presence of KN-93 and corrected by the EGFR expressed at the cell surface measured by FACS), after the addition of 10 nM EGF for the indicated time in DT40wt/EGFR (E) and ET1-55/EGFR (F) cells that were untreated (open circles) or treated with 1 µg/ml tetracycline for 24 (filled circles), 48 (filled squares) and 72 (filled triangles) hours previous to the assay. Error bars are shown if larger than the symbols. (G) The plot presents the mean ± SEM (n = 5) EGFR phosphorylation after the addition of 10 nM EGF for 2 min (triangles and right axis) and the level of CaM expression (circles and left axis) after incubation with 1 µg/ml tetracycline for the indicated time in DT40wt/EGFR (open symbols) and ET1-55/EGFR (filled symbols) cells. Error bars are shown if larger than the symbols.

FIGURE 4. Mutation of the CaM-binding domain of the EGFR decreases EGF-dependent EGFR activation and signaling. (A) Sequence of the CaM-binding domain of the wild type (wt) EGFR (amino acids 645-660) showing the three clusters of basic amino acids (highlighted in blue), and the A1A2 mutant EGFR where the six first basic amino acids were substituted by alanine (highlighted in green). (B) Serum-starved
HEK293 cells transiently transfected with either the vector coding for the human wild type EGFR (wt), the mutant EGFR (A1A2), and the empty vector (EV) were treated with 10 nM EGF for the indicated times and the reaction was arrested with ice-cold 10 % (w/v) trichloroacetic acid. Controls in the absence (-) of EGF were also included. The phosphorylated form of the EGFR (P-EGFR) was detected using an anti-phosphotyrosine antibody. (C) Serum-starved HEK293 cells transfected as above were treated with 10 nM EGF for 2 min. Controls in the absence (-) of EGF were also included. The phosphorylated (P-PLCγ1) and total (PLCγ1) phospholipase Cγ1 were detected using specific anti-phospho(Tyr1253)-PLCγ1 and anti-PLCγ1 antibodies. (D, E) Controls showing the total expression (D) and the expression at the plasma membrane (E) of the wild type EGFR (wt in panel D and red line in panel E), and the A1A2 mutant EGFR (A1A2 in panel D and blue line in panel E) and the absence of endogenous EGFR in mock cells transfected with the empty vector (EV), as determined using the anti-EGFR antibody. An anti-tubulin-α antibody was used as loading control (D), and as determined by FACS at the cell surface using an anti-EGFR antibody recognizing the extracellular region of the receptor. The gray peak represents background (Bckg) fluorescence. (F) The plot presents the mean ± SEM (n = 3) EGF-dependent phosphorylation of the EGFR and PLCγ in cells transfected with the wild type (red columns) and the A1A2 mutant (blue columns) receptors and stimulated with 10 nM EGF for 2-5 min, using the densitometric measurements of the phospho-EGFR/EGFR (total) and phospho-PLCγ/PLCγ (total) signal ratios from experiments similar to the ones presented in panels B-D.
Fig. 1 (Li et al. 2011)

A

B

C

D

E

F
Fig. 3 E-G (Li et al. 2011)
Fig. 4 (Li et al. 2011)

Panel A: Diagram showing the sequences of basic clusters 1, 2, and 3.

Panel B: Western blot analysis of EGFR phosphorylation in response to EGF treatment for varying time periods. 

Panel C: Comparison of EGFR phosphorylation in EV, wt, A1A2, wt, and A1A2 samples.

Panel D: Expression of PLCγ1 in EV, wt, A1A2, wt, and A1A2 samples.

Panel E: Flow cytometry analysis showing the expression of EGFR or PLCγ phosphorylation (%).

Panel F: Bar graph illustrating the wt (red) and A1A2 (blue) expression of P-EGFR and P-PLCγ.
Regulation of the ligand-dependent activation of the epidermal growth factor receptor by calmodulin
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