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The activating role of phospho-(Tyr)-calmodulin on the epidermal growth factor receptor

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The activity of calmodulin (CaM) is modulated not only by oscillations in the cytosolic concentration of free Ca^{2+}, but also by its phosphorylation status. In the present study, the role of tyrosine-phosphorylated CaM [P-(Tyr)-CaM] on the regulation of the epidermal growth factor receptor (EGFR) has been examined using *in vitro* assay systems. We show that phosphorylation of CaM by rat liver solubilized EGFR leads to a dramatic increase in the subsequent phosphorylation of poly-L-(Glu:Tyr) (PGT) by the receptor in the presence of ligand, both in the absence and in the presence of Ca^{2+}. This occurred in contrast with assays where P-(Tyr)-CaM accumulation was prevented by the presence of Ca^{2+}, absence of a basic cofactor required for CaM phosphorylation and/or absence of CaM itself. Moreover, an antibody against CaM, which inhibits its phosphorylation, prevented the extra ligand-dependent EGFR activation. Addition of purified P-(Tyr)-CaM, phosphorylated by recombinant c-Src (cellular sarcoma kinase) and free of non-phosphorylated CaM, obtained by affinity-chromatography using an immobilized anti-phospho-(Tyr)-antibody, also increased the ligand-dependent tyrosine kinase activity of the isolated EGFR toward PGT. Also a CaM(Y99D/Y138D) mutant mimicked the effect of P-(Tyr)-CaM on ligand-dependent EGFR activation. Finally, we demonstrate that P-(Tyr)-CaM binds to the same site (^{645}R-R-R-H-I-V-R-K-R-T-L-R-R-L-L-Q^{660}) as non-phosphorylated CaM, located at the cytosolic juxtamembrane region of the EGFR. These results show that P-(Tyr)-CaM is an activator of the EGFR and suggest that it could contribute to the CaM-mediated ligand-dependent activation of the receptor that we previously reported in living cells.

Key words: calcium, calmodulin, epidermal growth factor receptor, phospho-(Tyr)-calmodulin, tyrosine kinase.

INTRODUCTION

The epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein that belongs to the avian erythroblastosis oncogene B homolog (ErbB) receptor tyrosine kinase family and is implicated in the control of important cellular functions including cell proliferation, cell survival and apoptosis, differentiation and cell migration [1–3]. This receptor plays a prominent role in carcinogenesis and the growth of many solid tumours due to the occurrence in its encoding gene of point mutations, truncations, copy number gene amplification, overexpression and/or deregulated transactivation with distinct G protein-coupled receptors [4–7]. Therefore, the EGFR has been widely targeted for cancer therapy [8]. A general mechanism has been proposed to account for the activation of the EGFR upon ligand-induced dimerization. This consists in the asymmetric allosteric activation of the receptor when the C-terminal lobe of the kinase domain of one of the monomers interacts with the N-terminal lobe of the apposed monomer, thereby forming an activated dimer [9] or hetero-dimer with another ErbB family member.

Calmodulin (CaM) is a Ca^{2+}-receptor protein that transduces Ca^{2+}-mediated signals by binding to and regulating the activity of hundreds of enzymes and non-enzymatic proteins, thereby controlling a myriad of cellular functions [10–13]. EGFR signalling induces an early and transient increase in the cytosolic concentration of free Ca^{2+} [14–16], which results in the formation of the Ca^{2+}–CaM complex. We have previously shown that the Ca^{2+}–CaM complex binds to the EGFR *in vitro* and in living cells [17–23]. The CaM-binding domain (CaM-BD) of the receptor is located at its cytosolic juxtamembrane region [19,23–25] and binding of Ca^{2+}–CaM to this site exerts a positive triggering role in the ligand-dependent activation of the EGFR in living cells [21–23,25]. Likewise, ErbB2 also interacts with Ca^{2+}–CaM regulating downstream signalling [26]. Different mechanistic models have been proposed to account for the stimulatory action of Ca^{2+}–CaM on EGFR activation [27]. The most likely mechanism involves the Ca^{2+}–CaM-induced release of the positively-charged CaM-BD from the phosphoinositide-rich negatively-charged inner leaflet of the plasma membrane, an electrostatic interaction which otherwise would maintain the ligand-free receptor auto-inhibited [22,25,28]. Most significantly, the intracellular juxtamembrane region of the receptor, which contains the CaM-BD, has been shown to be indispensable for the allosteric activation mechanism mentioned above to be operative

Abbreviations: CaM, calmodulin; CaM-BD, CaM-binding domain; c-Src, cellular sarcoma kinase; DMEM, Dulbecco's modified Eagle's medium; DSS, disuccinimidyl suberate; EGFR, epidermal growth factor receptor; ErbB, avian erythroblastosis oncogene B homolog; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRP, horseradish peroxidase; NOS, nitric oxide synthase; P-(Tyr)-CaM, tyrosine-phosphorylated CaM; PDE1, phosphodiesterase 1; P-EGFR, auto(*trans*)-phosphorylated receptor; PGT, poly-L-(Glu:Tyr).

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[29–31], giving further credibility to the presumptive implication of CaM in the EGFR activation process. Although Ca^{2+}–CaM is involved in ligand-dependent EGFR activation [21–23,25], it is not clear whether or not phospho-(Tyr)-CaM (tyrosine-phosphorylated CaM) also plays an activating role in this process.

Distinct forms of CaM phosphorylated at serine, threonine or tyrosine residues have been shown to differentially modulate a variety of CaM-dependent target enzymes [32]. We have previously demonstrated that the EGFR phosphorylates CaM in the absence of Ca^{2+} and presence of different cationic polypeptides with a stoichiometry close to 1 (mol/mol), preferentially at Tyr^{99} as compared with Tyr^{138} [17,18,33–37]. Hence, we tested whether phospho-(Tyr)-CaM exerts an effect aimed at modulating the ligand-dependent activation of EGFR. In the present report, we show that tyrosine-phosphorylated CaM elicits a strong increase in the ligand-dependent activity of the EGFR *in vitro*, suggesting that phospho-(Tyr)-CaM may contribute to the ligand-dependent activation of this receptor in living cells, thereby enhancing EGFR-mediated signalling.

EXPERIMENTAL

Reagents

FBS and Dulbecco's modified Eagle's medium (DMEM) were obtained from Gibco. Radiolabelled [γ-^{32}P]ATP (triethylammonium salt; 3000–5000 Ci/mmol; 1 Ci = 37 GBq) and the ECL kit were purchased from Amersham and X-ray films were obtained from Amersham (Hyperfilm^{TM}-MP) or Eastman Kodak (X-Omat AR). Pre-stained molecular mass standards for protein electrophoresis were from Bio-Rad. CaM-agarose, ATP (sodium salt), calf thymus histone (type II-AS), Triton X-100, leupeptin (hemisulfate), Fast Green FCF, poly-L-(Glu:Tyr) (PGT; four glutamine–one tyrosine stoichiometry ratio; 44.5–45.7 kDa), disuccinimidyl suberate (DSS) and anti-mouse (Fc specific) IgG polyclonal (goat) antibody coupled to horseradish peroxidase (HRP) were purchased from Sigma–Aldrich. Mouse EGF (from submaxillary glands) was obtained from Sigma–Aldrich or Upstate Biotechnology and human recombinant EGF was from PeproTech EC. Pig brain CaM was purified essentially as previously described [38]. Mouse monoclonal anti-phosphotyrosine (clone 4G10, isotype $IgG_{2b\kappa}$) and anti-CaM (isotype IgG_1) antibodies were purchased from Millipore and rabbit monoclonal anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase; clone 14C10, isotype IgG) antibody was obtained from Cell Signaling Technology. Goat anti-rabbit IgG polyclonal antibody coupled to HRP was from Life Technologies. Other chemicals used in this work were of analytical grade.

Cell culture

Human carcinoma epidermoid A431 cells were obtained from the A.T.C.C. and were routinely cultured in DMEM supplemented with 10% (v/v) FBS, 2 mM L-glutamine and 40 μg/ml gentamicin at 37 °C in a humidified air atmosphere containing 5% CO_2.

Preparation of rat liver plasma membrane fraction and isolation of the EGFR

Liver plasma membrane fractions from young adult male Sprague–Dawley albino rats (200–250 g) were prepared at 4 °C following a method previously described [39] with some modifications as follows: the plasma membrane fraction isolated from the first sucrose gradient was used, the homogenization using a glass-Teflon homogenizer was increased up to 20 strokes and the 15 s homogenization with the Polytron was omitted. Leupeptin (2–5 μM) was added to the buffers to prevent proteolysis of the EGFR. To deplete the membranes of the Ca^{2+}-dependent bound CaM pool, 1 mM EGTA was added to all buffers and sucrose-gradient solutions. The membranes were finally resuspended in an EGTA-free buffer. The EGFR was isolated by Ca^{2+}-dependent CaM affinity chromatography essentially as described earlier [17]. Briefly, the membrane fraction was solubilized in a medium containing 25 mM Na–Hepes (pH 7.4), 5% (w/v) glycerol, 2–5 μM leupeptin 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 100 μM $CaCl_2$ was added to the supernatant just before loading the CaM-agarose column (4–5 ml of bed volume containing 5–6 mg of linked CaM) equilibrated with 25 mM Na–Hepes (pH 7.4), 5% (w/v) glycerol, 1% (w/v) Triton X-100 and 100 μM $CaCl_2$ (Ca^{2+}-buffer). After extensive washing with the Ca^{2+}-buffer, bound proteins were eluted with the same buffer containing 1 mM EGTA instead of $CaCl_2$ (EGTA-buffer) and 0.5–0.75-ml fractions were collected. We have found that it was very important to prepare the membranes in the presence of leupeptin and in the absence of added Ca^{2+} to prevent partial proteolysis of the EGFR by Ca^{2+}-dependent proteases. Therefore, $CaCl_2$ was added to the solubilized membranes just before loading the column. All chromatographic procedures were carried out at 4 °C.

Preparation of cell membrane fraction

A431 cells were washed with PBS (137 mM NaCl, 2.7 mM KCl, 12 mM Na/K-phosphate, pH 7.4), gently scraped from the plates, harvested by centrifugation and lysed with an homogenizer in 3 ml of an ice-cold hypotonic buffer containing 15 mM Hepes–NaOH (pH 7.4), 1 mM EGTA and a cocktail of protease inhibitors (0.5 mM AEBSF [4-(2-aminoethyl) benzenesulfonyl fluoride], 0.4 μM aprotinin, 25 μM bestatin, 7.5 μM E-64 {1-[*N*-[(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucyl]amino]-4-guanidinobutane}, 10 μM leupeptin, 5 μM pepstatin A and freshly prepared 0.6 mM PMSF). The lysate was incubated for 10 min on ice and centrifuged at 130 000 ***g*** for 30 min at 4 °C. The supernatant was discarded and the pellet resuspended in the same buffer and centrifuged as above. This pellet corresponding to the membrane fraction was washed with the same buffer but without EGTA, centrifuged again and resuspended in 3 ml of 25 mM Hepes–NaOH (pH 7.4) containing the protease inhibitors indicated above.

CaM phosphorylation and purification of ^{32}P-(Tyr)-CaM

CaM (50 μg) was phosphorylated at 37 °C for 2 h with purified human recombinant c-Src (cellular sarcoma kinase)(75 units) or the solubilized EGFR from rat liver in the presence of histone at a 1:1 ratio (mol/mol) in 1 ml of a medium containing 15 mM Hepes–NaOH (pH 7.4), 5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 0.2 mM Na_3VO_4, 50 mM NaF and 50 μM (20 μCi) [γ-^{32}P]ATP. One unit of c-Src transfers 2.6 pmoles of phosphate per hour to 375 μM of Src substrate peptide using the Src assay kit provided by the manufacturer. The formed ^{32}P-(Tyr)-CaM was purified as described earlier [36] in three steps: (i) isolation of the CaM fraction containing ^{32}P-(Tyr)-CaM and non-phosphorylated CaM using Ca^{2+}-dependent hydrophobic chromatography in a phenyl-Sepharose column; (ii) purification of ^{32}P-(Tyr)-CaM, free of non-phosphorylated CaM, by using a column of immobilized anti-phospho-tyrosine antibody covalently linked to agarose upon

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elution with phenyl-phosphate; and (iii) removal of phenyl-phosphate by filtration chromatography by using a Bio-Gel P-2 column [36]. Quantification of the purified ^{32}P-(Tyr)-CaM was done by measuring the densitometry of the band observed by Western blot using an anti-CaM antibody and known amounts of purified pig brain CaM as standards.

EGFR phosphorylation assays

The sequential two-step phosphorylation assays were run continuously without any pause between each step. In the first step, the EGF-activated EGFR phosphorylated CaM and in the second step phosphorylation of PGT by the receptor took place. Unless indicated otherwise the assays were performed as follows. First, CaM (0.7 μM) was phosphorylated by an EGFR preparation (50 μl) at 37 °C for 30 min in 100 μl of a medium containing 15 mM Hepes–NaOH (pH 7.4), 6 mM $MgCl_2$, 0.5 mM EGTA, 0.5 % (w/w) Triton X-100, 2.5 % (w/v) glycerol, 3 μM histone, 1 μM EGF (when added) and 100 μM (20 μCi) [γ-^{32}P]ATP. Thereafter, 0.6 mM $CaCl_2$ (100 μM free Ca^{2+}; when indicated) and 100 μg/ml PGT were added and the reaction was continued for times ranging from 1 to 10 min. Occasionally, 10 μM (2 μCi) [γ-^{32}P]ATP was used initially and a second identical pulse of 10 μM (2 μCi) [γ-^{32}P]ATP was added along with PGT to ascertain that enough radiolabelled ATP was available in the second part of the sequential assay. Controls were performed in the absence of CaM either by adding $CaCl_2$ at the start of the reaction or by omitting the addition of histone in order to prevent, in both cases, the phosphorylation of CaM. The receptor fraction was pre-incubated for 30 min on ice in the presence of EGF prior to phosphorylation. The reaction was initiated upon addition of radiolabelled ATP and stopped with ice-cold 10 % (w/v) trichloroacetic acid. The precipitated proteins were pelleted by centrifugation, the supernatant was discarded and the pellet processed by SDS/PAGE and autoradiography as described below. Alternatively, the phosphorylation of 100 μg/ml PGT by the EGFR preparation (50 μl) was performed at 37 °C for appropriate periods of time in 100 μl of a medium containing 15 mM Hepes–NaOH (pH 7.4), 6 mM $MgCl_2$, 0.5 mM EGTA, 0.5 % (w/w) Triton X-100, 2.5 % (w/v) glycerol and 30 nM histone, in the absence and presence of purified ^{32}P-(Tyr)-CaM (6 nM) free of non-phosphorylated CaM [histone/P-(Tyr)CaM molar ratio = 5], 1 μM EGF (when added) and 10 μM (2 μCi) [γ-^{32}P]ATP. The reaction was initiated and arrested as indicated above. When the phospho-mimetic mutant CaM(Y99D/Y138D) was tested, the detergent-solubilized EGFR from A431 cells was assayed in a reaction mixture containing 15 mM Hepes–NaO (pH 7.4), 2 mM $MgCl_2$, 2 mM EGTA or 100 μM $CaCl_2$, (depending of the conditions), 1 μM EGF (when added) and wild-type or mutant CaM (1 μg). The reaction was initiated upon addition of 2 mM ATP at 37 °C for 5 min and arrested upon addition of loading buffer. The samples were boiled at 100 °C for 5 min and processed for SDS/PAGE and Western blot using an anti-phospho-Tyr antibody to detect phospho-EGFR and an anti-GAPDH antibody as loading control, as detailed below.

Generation of the phospho-mimetic tyrosine–aspartic acid CaM mutant

The double mutant CaM(Y99D/Y138D) was prepared and purified as previously described [40]. Briefly, site-directed mutagenesis was performed using the QuickChange XL kit and as template the pETCM vector containing the coding sequence of the *Rattus norvegicus* CaM gene II [41]. The pair of complementary oligos used to generate the Y99D substitution was: 5′-GGCAATGGCGACATCAGTGCAGCA-3′ and 5′-TGCT GCACTGATGTCGCCATTGCC-3′. The product of this reaction was used as template for the Y138D substitution by using the following pair of complementary oligos: 5′-GGGGA TGGTCAGGTAAACGACGAAGAGTTTGTACAAATG-3′ and 5′-CATTTGTACAAACTCTTCGTCGTCGTTTACCTGACCAT CCCC-3′. The correctness of the mutagenesis procedure was ascertained by sequencing the resulting DNA inserts using an oligo annealing to the T7 promoter.

Expression and purification of recombinant proteins

Expression of wild-type CaM and the double mutant CaM(Y99D/Y138D) was done in *Escherichia coli* BL21(DE3)pLysS upon induction with 0.5 mM IPTG for 4 h and the recombinant proteins were purified to homogeneity as previously described [40]. The preparation and purification of the fusion protein between GST and the cytosolic juxtamembrane segment of the human EGFR comprising the amino acids (aa) 645–660 (GST–JM), corresponding to the CaM-BD of the receptor, was done, as previously described [19].

Cross-linkage assay

CaM/GST–JM cross-linkage assays were performed essentially as described [19]. The binding reaction (100 μl) contained 50 mM Hepes–NaOH (pH 7.4), 1 μg of GST–JM fusion protein, 50 μl of purified ^{32}P-(Tyr)-CaM (9400 cpm), 0.1 μg/ml PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin and either 0.27 mM $CaCl_2$ or 2 mM EGTA. Where indicated, a 10-fold molar excess of non-phosphorylated bovine brain CaM (3.4 μM), with respect to ^{32}P-(Tyr)-CaM (0.34 μM), was included. The reaction was allowed to proceed for 30 min at 37 °C and then the sample was incubated in the absence or presence of the cross-linking reagent DSS (3 mM) for 20 min on ice. The cross-linkage reaction was stopped upon addition of 250 mM glycine and further incubation on ice for 5 min. The proteins were precipitated by addition of ice-cold 10 % (w/v) trichloroacetic acid and after centrifugation at 13 000 ***g*** for 20 min the pellet was dissolved in SDS/PAGE loading buffer supplemented with 10 mM EGTA.

Electrophoresis, autoradiography and Western blot

Proteins were separated by slab SDS/PAGE in 5 %–20 % (w/v) linear gradient of polyacrylamide and 0.1 % (w/v) SDS at pH 8.3 as described [42] at 6 mA for 16–17 h. When required, 5–10 mM EGTA was added to the sample buffer to attain a lower mobility shift of CaM [43]. Gels were stained with Coomassie Brilliant Blue R-250, dried under vacuum at 70 °C–80 °C on Whatman 3MM Chr filter paper and blue-sensitive X-ray films were exposed at –20 °C for appropriate times. When required, the extent of the ^{32}P-labelled EGFR, CaM and PGT bands were quantified by scanning the autoradiographs using a computer-assisted photodensitometer. The photodensitometric intensities of the ^{32}P-labelled bands in the autoradiographs were directly proportional, within the exposure times used, to the amount of ^{32}P in the bands as measured in a scintillation counter. Alternatively, after SDS/PAGE the proteins were electrotransferred from the gel to a PVDF membrane for 2 h at 300 mA in a medium containing 48 mM Tris-base, 36.6 mM L-glycine, 0.04 % (w/v) SDS and 20 % (v/v) methanol. The proteins were fixed with 0.2 % (v/v) glutaraldehyde in 25 mM Tris/HCl (pH 8), 150 mM NaCl and 2.7 mM KCl for 45 min and transiently stained with Fast Green

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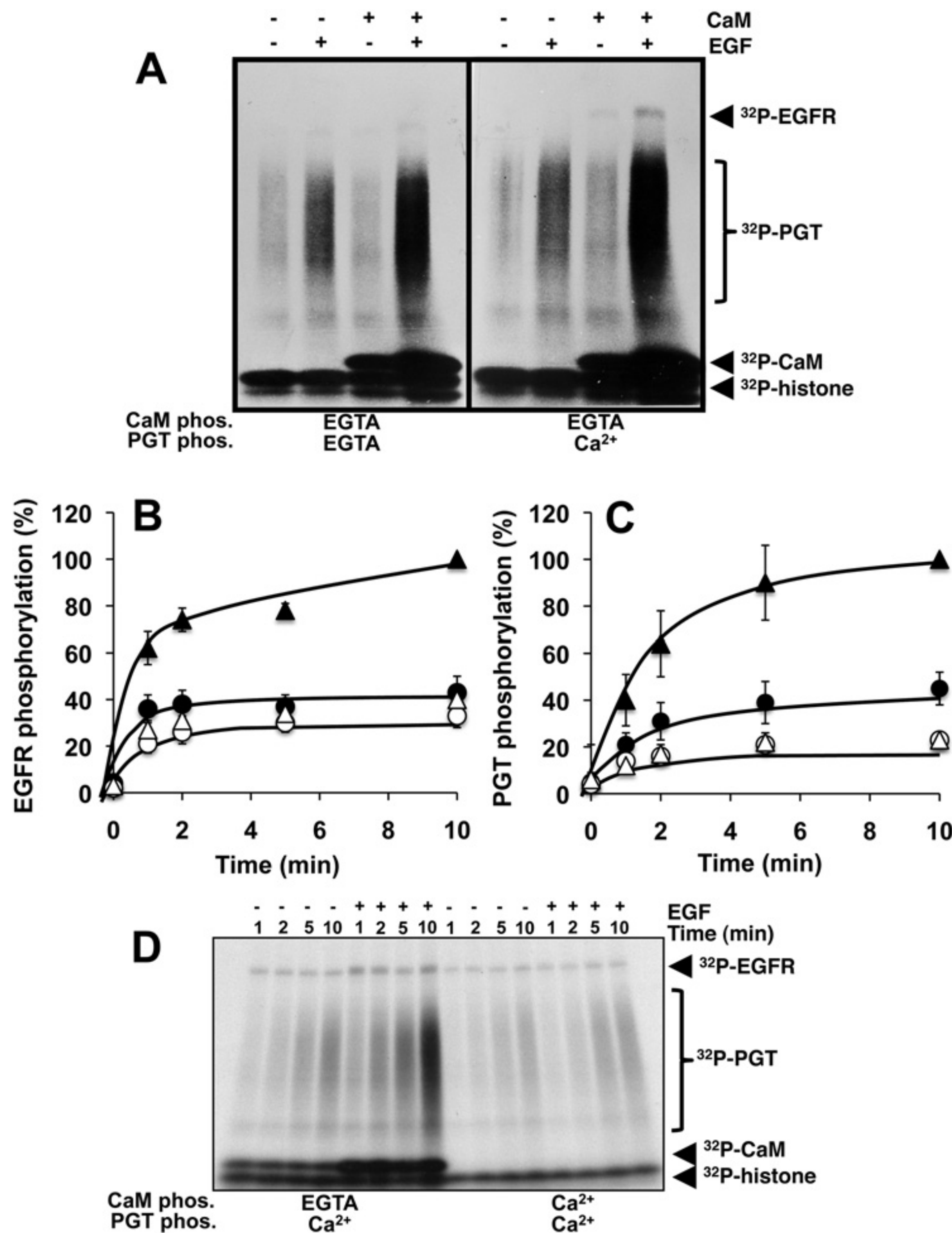

Figure 1 Phospho-(Tyr)-CaM enhances the EGF-dependent tyrosine kinase activity of the EGFR

(**A**) CaM (0.7 μg) was phosphorylated (CaM phos.) with a purified rat liver EGFR preparation for 30 min in the presence of 0.5 mM EGTA, either in the absence (–) or presence (+) of 1 μM EGF. In a second step of the same assay, 100 μg/ml PGT was added and phosphorylated for 5 min (PGT phos.). This phosphorylation step was carried out in the absence of free Ca^{2+} (EGTA) or in its presence (Ca^{2+}) as indicated. Controls in the absence of CaM were included. The arrowheads point to the phosphorylated form of the receptor (^{32}P-EGFR), CaM (^{32}P-CaM) and histone (^{32}P-histone), and the bracket phosphorylated PGT (^{32}P-PGT). The plots present the mean $\pm$ S.E.M. time-course phosphorylation of the EGFR (**B**) and PGT (**C**) from five independent experiments similar to the one shown in (**A**), carried out in the absence (open symbols) or presence (filled symbols) of 1 μM EGF and in the absence (circles) or presence (triangles) of CaM. (**D**) The sequential assay was performed as indicated in (**A**), except that CaM was added to all the samples and its phosphorylation was allowed to proceed only in the absence of Ca^{2+} (EGTA) or prevented by its presence (Ca^{2+}) and the subsequent phosphorylation of PGT was carried out in the absence or presence of EGF for the indicated times in the presence of 100 μM free Ca^{2+}.

to assess the regularity of the transfer procedure. The PVDF membranes were blocked with 5 % (w/v) bovine serum albumin or 5 % (w/v) fat-free powdered milk, following the instructions of the antibodies' manufacturers, in 0.1 % (w/v) Tween-20, 100 mM Tris/HCl (pH 8.8), 500 mM NaCl and 0.25 mM KCl and probed 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 a secondary anti-IgG antibody coupled to HRP. The bands were visualized upon development by ECL and exposure of X-ray films for appropriate periods of time. The intensity of the bands was quantified using the ImageJ 1.46r program.

Other analytical procedures

Protein concentration was determined by the Lowry [44] or the bicinchoninic acid [45] methods using BSA as a standard. The concentration of free Ca^{2+} in the assay system was calculated by using a computer program similar to the one previously described [46].

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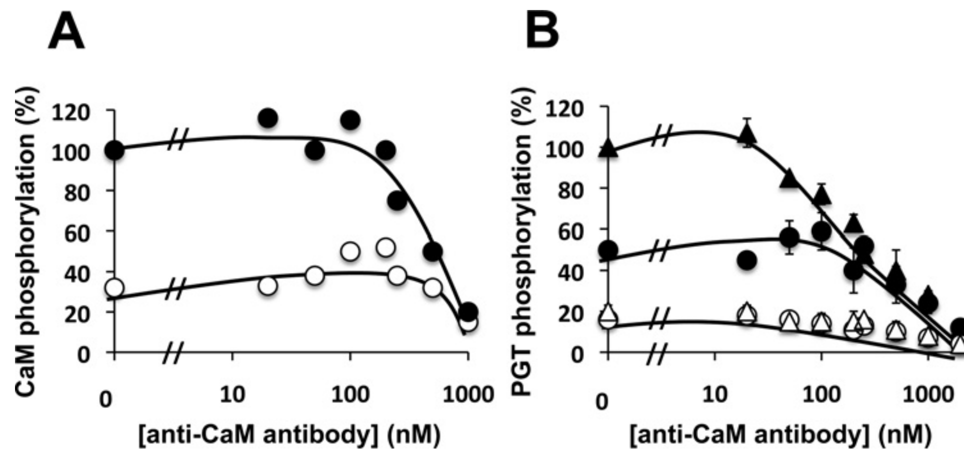

Figure 2 An anti-CaM antibody inhibits EGFR-mediated CaM phosphorylation and decreases the extra EGF-dependent activation of the receptor

CaM (0.7 μg) was incubated with the indicated concentration of a monoclonal anti-CaM antibody for 1.5 h on ice and phosphorylated in a first step for 5 min in the absence (open symbols) or presence (filled symbols) of 1 μM EGF using a purified preparation of rat liver EGFR. Thereafter, 100 μg/ml PGT was added to the same assay and its phosphorylation level in the presence of Ca^{2+} was determined at 1.5 min as described in Figure 1(A). The plots present the phosphorylation of CaM (**A**) and the mean $\pm$ range phosphorylation of PGT (**B**) from two independent experiments.

Statistical analysis

The two-tailed paired Student's t test was performed using the Microsoft Excel (Microsoft Co.) software. Data are expressed as the mean $\pm$ S.E.M. and differences are considered significant at $P \leqslant 0.05$.

RESULTS

EGFR-phosphorylated CaM enhances EGF-dependent EGFR activation

Testing the effect of P-(Tyr)-CaM on EGFR activation in living cells is a challenging task, since the amount of P-(Tyr)-CaM in most non-transformed cells and tissues appears to be very low or undetectable, in contrast with the more abundant P-(Ser/Thr)-CaM species [47–49]. We therefore tested the effect of P-(Tyr)-CaM on the EGFR using an *in vitro* assay system. In a first approach, we designed a sequential assay system in which the EGF-activated EGFR first phosphorylated CaM in the absence of Ca^{2+} and presence of histones and thereafter the tyrosine kinase activity of the receptor toward the exogenous substrate PGT was determined. Figure 1(A) shows the phosphorylation of CaM in the absence and presence of EGF, performed in the first step of the assay in the absence of Ca^{2+} (presence of EGTA) as indicated and the phosphorylation of PGT, subsequently assayed in the second step within the same reaction mixture either in the absence of Ca^{2+} (EGTA) or in its presence (Ca^{2+}). As we have previously reported, the addition of Ca^{2+} just before starting the phosphorylation of PGT in the second step should prevent further phosphorylation of CaM by the EGFR [17,18,33,34]. It can be observed that the phosphorylation of PGT, detected as a smear along the electrophoretic tracks, was higher when P-(Tyr)-CaM became accumulated in the first part of the sequential assay, as compared to control experiments performed in the absence of CaM. This activation was not observed in the absence of EGF. The Ca^{2+}-induced electrophoretic mobility shift of accumulated P-(Tyr)-CaM at 21 kDa was attained by including EGTA in the electrophoresis sample buffer [43], which allowed ^{32}P-(Tyr)-CaM to be resolved from the ^{32}P-histone bands. The auto(*trans*)-phosphorylated receptor (P-EGFR) was also visible as a ^{32}P-labelled 170 kDa band. Figures 1(B) and 1(C) show a time-course of the auto(*trans*)-phosphorylation of the EGFR and the phosphorylation of PGT in assays performed in the absence (open symbols) or presence (filled symbols) of EGF and in the absence (circles) or presence (triangles) of accumulated P-(Tyr)-CaM, in experiments similar to that presented in Figure 1(A) except that the time of reaction was varied as indicated. As is apparent, the presence of P-(Tyr)-CaM, accumulated during the first step of the sequential assay, enhanced both the auto(*trans*)-phosphorylation of the receptor and the phosphorylation of PGT 2.5–3-fold in the presence of EGF, with little if any effect in its absence.

To ascertain that the ligand-dependent extra-activation observed on the EGFR tyrosine kinase was due to the presence of P-(Tyr)-CaM, additional experiments were performed in the presence of CaM, but in which its phosphorylation during the first step of the assay was prevented by the presence of Ca^{2+}, as this cation acts as an inhibitor of CaM phosphorylation as previously demonstrated [17,18,33,34]. As can be observed in Figure 1(D), in the presence of Ca^{2+} P-(Tyr)-CaM was not accumulated and the subsequent extra activation of the EGFR in the presence of EGF was absent. This contrasted with the strong activation observed when CaM was phosphorylated in the absence of free Ca^{2+} (presence of EGTA) during the first step of the assay.

Histone acts as a cationic cofactor for the phosphorylation of CaM and given that, the amount of P-(Tyr)-CaM formed in the assay depends on the CaM–histone ratio used [33,34]. Upon changing this ratio, we observed that the activation of the EGFR in the presence of EGF was directly proportional to the amount of accumulated P-(Tyr)-CaM (result not shown). Moreover, we tested CaMs isolated from *Trypanosoma cruzi* and *Leishmania mexicana*, which lack Tyr^{99} and are not phosphorylated by the EGFR [35]. In these cases no EGF-dependent extra-activation of the EGFR tyrosine kinase was observed due to the absence of P-(Tyr)-CaM in the system (result not shown).

An anti-CaM antibody inhibits CaM phosphorylation and subsequent EGF-dependent EGFR activation

To further ascertain that the observed extra EGF-dependent activation of the EGFR was mediated by the accumulated P-

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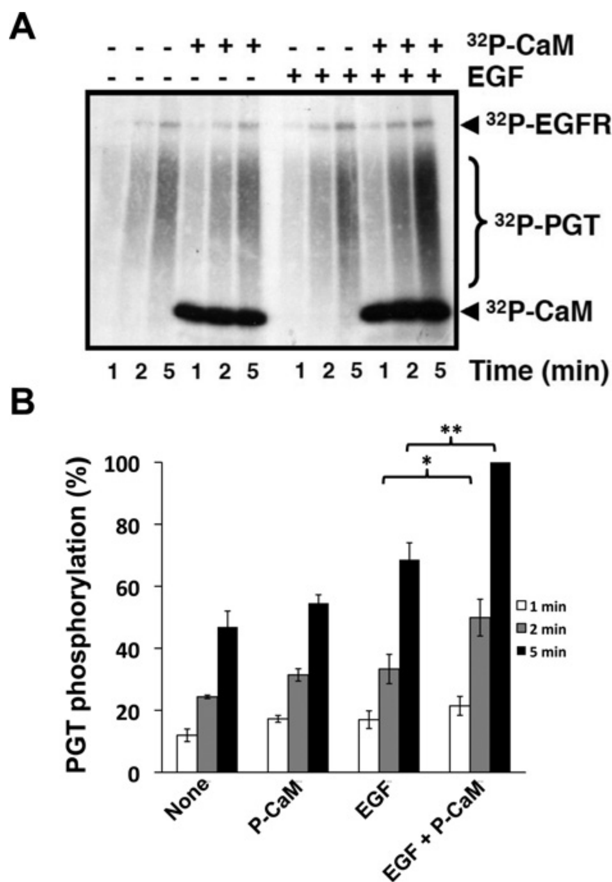

Figure 3 Purified phospho-(Tyr)-CaM enhances the EGF-dependent tyrosine kinase activity of the EGFR

(**A**) Phosphorylation of 100 μg/ml PGT by a purified rat liver EGFR preparation was assayed in the absence (–) or presence (+) of 1 μM EGF, 30 nM histone and purified P-(Tyr)-CaM (6 nM) [histone/P-(Tyr)-CaM molar ratio = 5] for the indicated times. The reaction was initiated upon addition of 10 μM (2 μCi) [γ-^{32}P]ATP and stopped with ice-cold 10 % (w/v) trichloroacetic acid. Arrowheads point to the phosphorylated forms of the receptor (^{32}P-EGFR) and CaM (^{32}P-CaM) and the bracket indicates phosphorylated PGT (^{32}P-PGT). (**B**) The plot presents the mean ± S.E.M. phosphorylation levels of PGT attained at the indicated times from three independent experiments in which histone was either absent or a histone–P-(Tyr)-CaM ratio (mol/mol) of 1 or 5 was used. No significant differences were found between the experiments performed in the absence or presence of histone. $^{*}P < 0.05$ and $^{**}P < 0.01$ as determined by the Student's *t* test.

(Tyr)-CaM in the first part of the sequential assay system described above, the effect of a monoclonal antibody against CaM was tested. Figure 2 shows that the addition of progressively higher concentrations of this antibody gradually inhibited the phosphorylation of CaM (Figure 2A) in the presence (filled circles) and absence (open circles) of EGF in the first step of the sequential assay and the EGF-dependent phosphorylation of PGT measured in the second step of the same assay (Figure 2B). The inhibitory effect of the anti-CaM antibody was primarily directed to the extra EGF-dependent activation of the EGFR, as is clearly observed in the assays in which P-(Tyr)-CaM was accumulated (filled triangles), as compared with assays in the presence of EGF but in the absence of accumulated P-(Tyr)-CaM (filled circles). In the absence of EGF the phosphorylation of PGT was very low as expected and no difference in the absence (open circles) compared with presence (open triangles) of P-(Tyr)-CaM was detected.

Purified P-(Tyr)-CaM enhances EGF-dependent EGFR activation

To directly test the potential capacity of P-(Tyr)-CaM to enhance the EGF-dependent activation of the isolated EGFR, we phosphorylated CaM with recombinant c-Src *in vitro* and prepared purified P-(Tyr)-CaM free of non-phosphorylated CaM by using the method previously described [36]. Figure 3(A) shows that addition of purified ^{32}P-(Tyr)-CaM to the assay system strongly enhanced the EGF-dependent phosphorylation of PGT mediated by the EGFR, whereas no significant effect of ^{32}P-(Tyr)-CaM was detected in the absence of EGF. Figure 3(B) shows a time-course of the EGF-dependent phosphorylation of PGT in which higher phosphorylation of PGT in the presence of ^{32}P-(Tyr)-CaM than in its absence was observed. Moreover, this extra activation was not observed in the absence of EGF. We calculated that the extent of the EGF-dependent phosphorylation of PGT (subtracting the phosphorylation in the absence of EGF from that in the presence of EGF) was of ∼ 2.1-fold higher in the presence of ^{32}P-(Tyr)-CaM than in its absence, as determined by densitometry of the ^{32}P-PGT smear in the autoradiogram (result not shown).

The CaM(Y99D/Y138D) mutant mimics the effect of P-(Tyr)-CaM on EGF-dependent EGFR activation

We also tested the effect of the phospho-mimetic CaM(Y99D/Y138D) double mutant on the EGF-dependent activation of the solubilized EGFR, as compared with wild-type CaM. Figure 4(A) shows that the EGF-dependent phosphorylation level of the EGFR was higher in the presence of the mutant than of wild-type CaM, both in the absence of Ca^{2+} (presence of EGTA) and in its presence. Figure 4(B) shows the quantification of this effect in a series of experiments similar to the one shown above (Figure 4A). It can be observed that CaM(Y99D/Y138D) elicited a ∼2-fold increment as compared with wild-type CaM in the presence of Ca^{2+} (open bars), whereas a lower but significant increment of approximately 40 % was observed in the absence of Ca^{2+} (presence of EGTA; solid bars).

P-(Tyr)-CaM and non-phosphorylated CaM both bind to the cytosolic juxtamembrane region of the EGFR

We have previously demonstrated that the CaM-BD of the EGFR is located at the cytosolic juxtamembrane region, comprising the sequence ^{645}R-R-R-H-I-V-R-K-R-T-L-R-R-L-L-Q^{660} [19]. To determine whether P-(Tyr)-CaM binds to the same region as non-phosphorylated CaM, we performed cross-linking experiments using purified ^{32}P-(Tyr)-CaM and a GST fusion protein containing the indicated CaM-BD of human EGFR (GST–JM). Figure 5 shows that in the presence of the cross-linking agent DSS, but not in its absence, the ^{32}P-CaM/GST–JM complex was detected (∼50 kDa) in the presence of Ca^{2+}. By contrast, in the absence of Ca^{2+} (presence of EGTA) this signal was not detected. Moreover, the addition of a 10-fold excess of non-phosphorylated CaM chased the binding of ^{32}P-(Tyr)-CaM to GST–JM. This demonstrates that both non-phosphorylated CaM and P-(Tyr)-CaM bind to the same cytosolic juxtamembrane site of the EGFR in a Ca^{2+}-dependent manner.

DISCUSSION

The transient increase in the concentration of cytosolic free Ca^{2+} upon cell stimulation by hormones or mitogenic factors favours the formation of the Ca^{2+}–CaM complex [50], in turn regulating a myriad of CaM-dependent proteins [10–13]. Nevertheless, it

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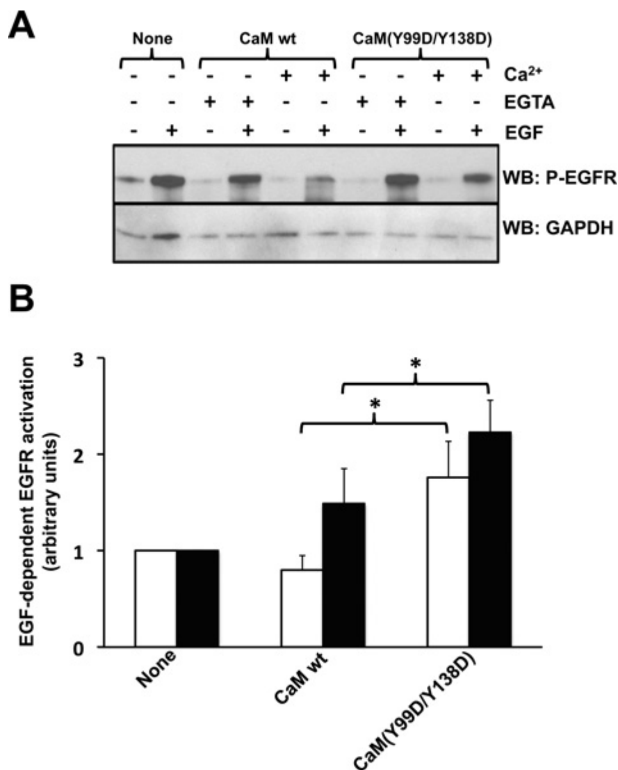

Figure 4 The phospho-mimetic mutant CaM(Y99D/Y138D) enhances the EGF-dependent activation of the EGFR as compared with wild-type CaM

(**A**) EGFR solubilized from a membrane fraction of serum-starved A431 cells was incubated for 5 min in the absence (none) or presence of the indicated CaM species (1 μg) in the absence (–) of Ca^{2+} (presence of EGTA) or in its presence (+) and in the absence (–) or presence (+) of 1 μM EGF as indicated. The samples were processed by SDS/PAGE and Western blot (WB) using an anti-phospho-Tyr antibody. GAPDH is shown as a loading control. (**B**) The histogram presents the mean $\pm$ S.E.M. EGF-dependent EGFR activity in the absence (solid bars) or presence (open bars) of Ca^{2+} and the different CaM species determined by measuring the P-EGFR/GAPDH ratio from six independent experiments. $^{*}P < 0.05$ as determined by the Student's *t* test. wt, wild-type.

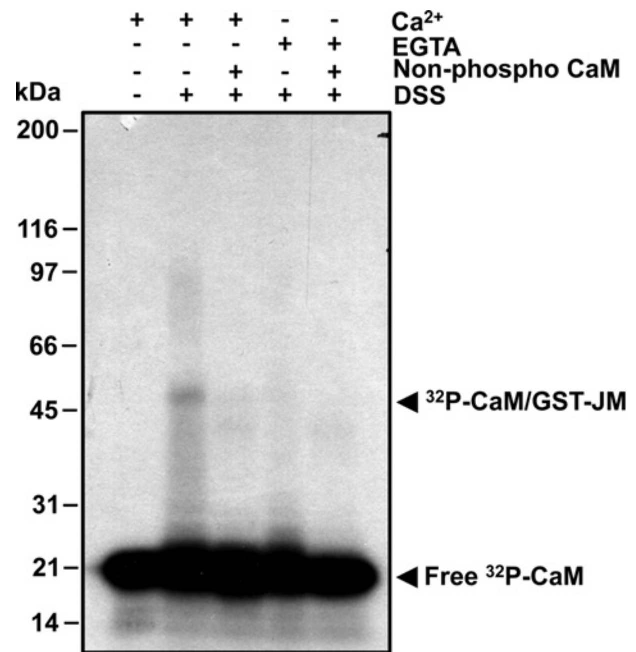

Figure 5 Phospho-(Tyr)-CaM and non-phosphorylated CaM both bind to the cytosolic juxtamembrane region of the EGFR

The GST fusion protein containing the CaM-BD of the EGFR (GST-JM) and purified ^{32}P-(Tyr)-CaM, free of non-phosphorylated CaM, were cross-linked with 3 mM DSS in the presence of either 0.27 mM $CaCl_2$ or 2 mM EGTA and where indicated a 10-fold excess of non-phosphorylated CaM (non-phospho CaM). A control in the absence of DSS is also presented. Arrowheads point to the ^{32}P-CaM–GST-JM complex and non-reacted ^{32}P-(Tyr)-CaM.

is reasonable to assume that Ca^{2+} may not be the only signal with the capacity to modulate the CaM-dependent systems in the cell. A wide range of serine/threonine- and tyrosine-protein kinases have been shown to phosphorylate CaM *in vitro* and in living cells. The different phospho-CaM species have different regulatory roles in the activity of many CaM regulated proteins as compared with non-phosphorylated CaM [32]. This suggests that this post-translational modification, in addition to Ca^{2+} binding, could modulate the activity of CaM.

The non-receptor tyrosine kinase Src [48], the insulin receptor [51] and the EGFR [33], are kinases involved in the phosphorylation of CaM at both Tyr[99] and Tyr[138]. The dynamic nature of this process and the distinct phosphorylated forms of CaM at a given time could be difficult to track in living cells due to the short half-life of P-(Tyr)-CaM. Thus, the potential differential role exerted by P-(Tyr)-CaM as compared with non-phosphorylated CaM has been assessed *in vitro* on systems such as phosphodiesterase 1 (PDE1), nitric oxide synthase (NOS) and the plasma membrane Ca^{2+}-ATPase among others [32]. CaM phosphorylated by the EGFR was incapable of activating PDE1 from bovine heart [36], whereas insulin receptor-phosphorylated CaM has no differential effect as compared to non-phosphorylated CaM when tested toward PDE1 isolated from rat hepatocytes [52]. P-(Tyr99)-CaM tested on NOS had a tremendous effect on its activity, increasing the V_{max} of the enzyme more than 3-fold compared with non-phosphorylated CaM [53]. It has also been reported that P-(Tyr)-CaM is required for the activation of the Na^{+}/H^{+} exchanger type 1 (NHE1) in podocytes through a mechanism involving EGFR and Janus kinase 2 (JAK2) [54]. Overall, the tyrosine and serine/threonine phosphorylated site(s) of CaM, the latter not discussed in the present study, as well as the phosphorylation stoichiometry, appear to be relevant to modulate the activity of different CaM-dependent systems when comparing non-phosphorylated CaM with its phosphorylated counterparts [32].

CaM is more efficiently phosphorylated at serine/threonine than at tyrosine residues in living cells [47–49]. Thus, to determine whether P-(Tyr)-CaM could be involved in the EGF-dependent activation of the EGFR, we set to assay *in vitro* the isolated EGFR from rat liver and A431 cells. Using the isolated EGFR we clearly show in the present work that P-(Tyr)-CaM enhances the basal EGF-dependent activation of the EGFR both in the absence and in the presence of Ca^{2+}, in contrast with the inhibitory effect that non-phosphorylated CaM exerts on the receptor in the presence, but not in the absence of Ca^{2+}, when assayed *in vitro* [17,18]. In our assays, we first tested the extra activating effect of accumulated P-(Tyr)-CaM, previously phosphorylated by the EGFR, in a sequential assay system. Second, we assayed the extra activating effect of purified P-(Tyr)-CaM, phosphorylated by recombinant c-Src and free of non-phosphorylated CaM. In both assays we obtained identical results. Although the relative extent of CaM phosphorylation at Tyr[99] and Tyr[138] could vary using the two kinases (EGFR and c-Src), we have previously shown using a set of CaM mutants harbouring tyrosine to phenylalanine [37] or tyrosine to aspartic acid/glutamic acid [40] substitutions, that both

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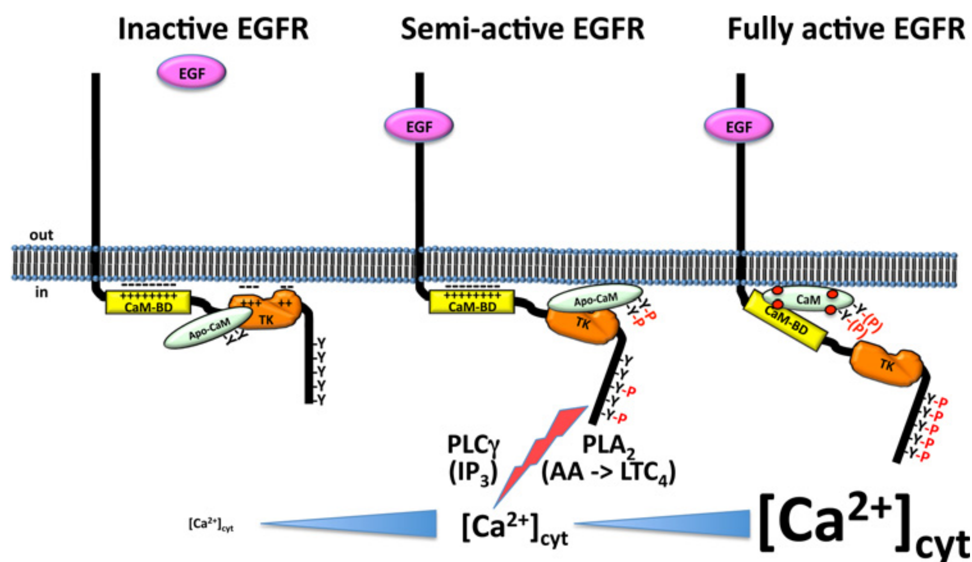

Figure 6 Potential role of phospho-(Tyr)-CaM in ligand-dependent EGFR activation

(Left) The cytosolic concentration of free Ca^{2+} is very low in resting conditions and both the CaM-BD and the tyrosine kinase domain (TK) of the ligand-free EGFR are electrostatically bound to the inner leaflet of the plasma membrane promoting its auto-inhibition as proposed by McLaughlin et al. [25] and apo-CaM can be tethered to the receptor close to its TK. (Centre) Upon EGF binding, the receptor is partially activated, apo-CaM is phosphorylated and phospho-(Tyr)-CaM help to detach the TK from the membrane. This initiates an increase in the cytosolic concentration of free Ca^{2+} via activation of phospholipase Cγ (PLCγ) and phospholipase A_2 (PLA_2) with the ensuing formation, respectively, of the Ca^{2+}-mobilizing agent inositol-1,4,5-trisphosphate (IP_3) and arachidonic acid (AA), which is metabolized to leukotriene C_4 (LTC_4), an additional Ca^{2+}-mobilizing compound. (Right) When the cytosolic concentration of free Ca^{2+} is high enough the Ca^{2+}–CaM and/or Ca^{2+}/phospho-(Tyr)-CaM complexes are formed, binding to the CaM-BD of the EGFR that is detached from the membrane, and achieving full activation of the receptor. For simplicity only a monomer of the EGF-bound receptor dimer is depicted.

kinases are able to phosphorylate both tyrosine residues, although c-Src phosphorylates Tyr^{138} more efficiently than Tyr^{99}.

Apparently, non-phosphorylated CaM only interacts with the EGFR when is not phosphorylated at Tyr^{1173}, while failing to interact with the receptor phosphorylated at this residue. In contrast, P-(Tyr138)-CaM interacts with the EGFR in both non-phosphorylated and phosphorylated states [55]. We have also demonstrated that both non-phosphorylated CaM and P-(Tyr)-CaM bind to the same cytosolic juxtamembrane region of the EGFR (aa 645–660). Thus, it is likely that both CaM species may bind to this site in living cells. The identification of P-(Tyr)-CaM as a potential intracellular ligand of the EGFR introduces further complexity to the proposed activation mechanism of this receptor [9,29–31]. Thus, we suggest the existence of three fundamental states of the EGFR tyrosine kinase: (i) an inactive state in the absence of EGF in which the CaM-BD is electrostatically bound to the inner leaflet of the plasma membrane as first proposed by McLaughlin et al. [25]; (ii) a partially-active state of the receptor in the presence of EGF when apo-CaM is phosphorylated and P-(Tyr)-CaM detaches the tyrosine kinase domain from the membrane; and (iii) a fully-active state in the presence of EGF when Ca^{2+}/P-(Tyr)-CaM plus non-phosphorylated Ca^{2+}–CaM are present, as the former plays a more efficient activating role and the latter may also accumulate after Ca^{2+}/P-(Tyr)-CaM dephosphorylation. This suggests that there may exist a three-gear modulatory mechanism built into the receptor to modulate EGF-dependent signalling.

To integrate within a physiological framework the transient EGF-dependent Ca^{2+} signal generated by the receptor [27,56] with the positive regulatory effect of P-(Tyr)-CaM on the EGF-dependent EGFR activation (the present work), we propose a hypothetical CaM/P-(Tyr)-CaM regulatory cycle whose main steps could be described as follows (Figure 6). (i) In the absence of ligand the EGFR is auto-inhibited; (ii) upon EGF stimulation, the low cytosolic concentration of free Ca^{2+} initially prevailing under such conditions [57] could favour an ultra-fast phosphorylation of apo-CaM by the EGFR [17,18,33,34], particularly if CaM were to be already tethered to the receptor; (iii) the generated P-(Tyr)-CaM could help to partially activate the EGFR, initiating the generation of the Ca^{2+} signal; (iv) as the cytosolic concentration of free Ca^{2+} rises [56], additional phosphorylation of CaM should come to a halt [17,18,33,34]; and (v) the increase in the cytosolic concentration of free Ca^{2+} would induce the formation of the Ca^{2+}–CaM and/or Ca^{2+}/P-(Tyr)-CaM complexes allowing the complete detachment of the CaM-BD of the EGFR from the inner leaflet of the plasma membrane according to the model proposed by McLaughlin et al. [25], thereby maintaining the receptor in a fully active state.

If this hypothetical model were substantiated in living cells, an additional important issue to be addressed could be the identification of the tyrosine-phosphatase(s) involved in the dephosphorylation of P-(Tyr)-CaM and to determine whether or not such phosphatase(s) would exhibit a Ca^{2+} requirement. The existence of a CaM/P-(Tyr)-CaM cycle involved in the regulation of the EGFR could have important consequences for the control of cell proliferation, as its alteration could potentially result in uncontrolled cell proliferation. Therefore, a comparative study of this system in tumour compared with non-transformed cells could yield further insight into validating the possible physiological relevance of this regulatory mechanism.

AUTHORS CONTRIBUTION

Silviya Stateva, Valentina Salas, Alberto Benguría, Itziar Cossío, Estefanía Anguita and José Martín-Nieto performed the experiments. Antonio Villalobo and Gustavo Benaim supervised the experiments and all authors analysed the results. A.V. designed the project and wrote the paper.

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