Phosphorylation of Calmodulin by Permeabilized Fibroblasts Overexpressing the Human Epidermal Growth Factor Receptor

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Detergent-permeabilized EGFR-T17 fibroblasts, which overexpress the human epidermal growth factor (EGF) receptor, phosphorylate both poly-L-(glutamic acid, tyrosine) and exogenous calmodulin in an EGF-stimulated manner. Phosphorylation of calmodulin requires the presence of cationic polypeptides, such as poly-L-(lysine) or histones, which exert a biphasic effect toward calmodulin phosphorylation. Optimum cationic polypeptide/calmodulin molar ratios of 0.3 and 7 were determined for poly-L-(lysine) and histones, respectively. Maximum levels of calmodulin phosphorylation were attained in the absence of free calcium, and a strong inhibition of this process was observed at very low concentrations ($K_i = 0.2\,\mu M$) of this cation. The incorporation of phosphate into calmodulin occurred predominantly on tyrosine residue(s) and was stimulated 34-fold by EGF.

Key words: Cell permeabilization / Phosphocalmodulin / Tyrosine kinase.

Introduction

The epidermal growth factor receptor (EGFR) is an integral membrane tyrosine kinase glycoprotein of 170 kDa involved in cell proliferation and differentiation processes in a variety of cell types. Signalling by this receptor involves its transphosphorylation and the phosphorylation of intracellular target proteins, which commits the cell to its mitotic division (Carpenter, 1987; Schlessinger, 1988; Ullrich and Schlessinger, 1990; Fantl *et al.*, 1993). Additionally, one of the early events occurring during the transduction of the mitogenic signal mediated by the EGFR is an increase in the cytosolic concentration of free Ca^{2+} (Moolenaar *et al.*, 1986; Pandiella *et al.*, 1988).

A number of Ca^{2+}-dependent enzymes and cellular processes have been shown to be modulated by the intracellular Ca^{2+} receptor protein, calmodulin (Means and Dedman, 1980; Klee and Vanaman, 1982; Bachs *et al.*, 1992; James *et al.*, 1995), including the regulation of cell division (Veigl *et al.*, 1984; Reddy *et al.*, 1994; Bachs *et al.*, 1994; Takuwa *et al.*, 1995). Additional experimental evidence obtained in our laboratory supports the involvement of calmodulin in the regulation of cell proliferation (San José *et al.*, 1992; Benguría and Villalobo, 1993; Benguría *et al.*, 1994, 1995a). First, the EGFR is isolated from solubilized rat liver plasma membranes by Ca^{2+}-dependent calmodulin-affinity chromatography. Second, the isolated receptor phosphorylates exogenous calmodulin on tyrosine-99 with a stoichiometry close to 1, in the absence of Ca^{2+} and presence of a cationic polypeptide as cofactor. Third, calmodulin inhibits both the EGFR transphosphorylation and EGFR-mediated phosphorylation of exogenous substrates in a Ca^{2+}-dependent manner. Taken together, these results led us to postulate that calmodulin exerts a regulatory role on the EGFR tyrosine kinase activity (San José *et al.*, 1992; Benguría and Villalobo, 1993; Benguría *et al.*, 1994; 1995a).
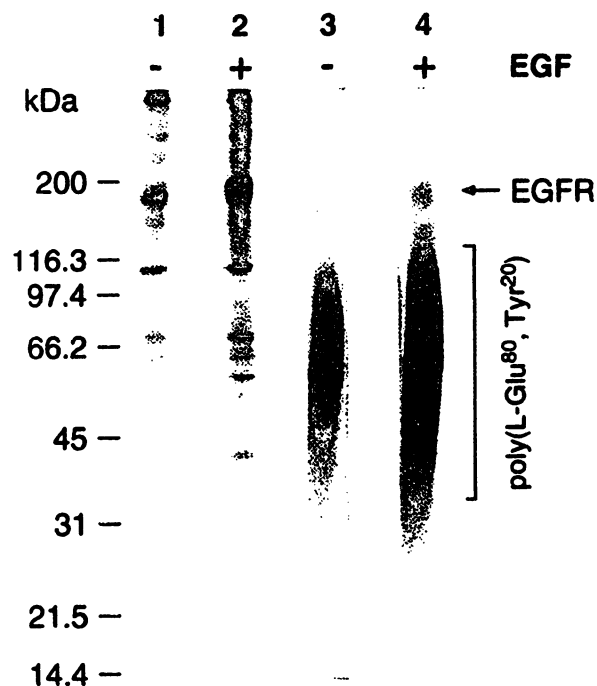
In this work we demonstrate that exogenous calmodulin is phosphorylated *in vitro* in an EGF-stimulated manner by permeabilized fibroblasts overexpressing the EGFR. This phosphorylation process exhibits similar properties to those determined for the isolated EGFR from rat liver (San José *et al.*, 1992; Benguría and Villalobo, 1993; Benguría *et al.*, 1994, 1995a), which suggests that EGF-mediated phosporylation of calmodulin may be a generalized process among mammalian cells.

Results and Discussion

Assay of the Epidermal Growth Factor Receptor Tyrosine Kinase Activity in Permeabilized Cells

The EGFR-T17 fibroblast cell line is an NIH 3T3-derived clone which has been stably transfected with a human EGFR cDNA using a retroviral expression system (Velu *et al.*, 1987; Pandiella *et al.*, 1988). These cells overexpress high affinity ($K_d = 3-5\,nM$) human EGFR, at a number of 4×10^5 receptors/cell as estimated by [^{125}I]EGF binding assays, and develop a transformed phenotype in the presence of EGF in the culture medium (Velu *et al.*, 1987; Pandiella *et al.*, 1988), thus constituting a valuable material for the study of signalling processes mediated by the EGFR (Pandiella *et al.*, 1988, 1989; Magni *et al.*, 1991).

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To test whether the EGFR-T17 fibroblasts would constitute a suitable material for the assay of EGFR tyrosine kinase activity, we performed *in vitro* phosphorylation assays in which the cells were permeabilized with 0.1% (w/v) Triton X-100 prior to the addition of [γ-^{32}P]ATP. This treatment preserved the boundary and the gross organization of the cell, as assessed by phase-contrast light microscopy (results not shown). Using this assay system we have found that the addition of 1 μM EGF resulted in a 4.3 ± 0.8 (mean ± S.E.M., n = 7)-fold increase in the phosphorylation of a 170 kDa band, as shown in Figure 1 (lanes 1 and 2). The identity of this band as the phosphorylated EGFR was confirmed through covalent cross-linkage of [^{125}I]EGF to the 170 kDa protein in whole non-permeabilized cells (results not shown). Furthermore, we have previously shown that the 170 kDa phosphoprotein is immunoprecipitated from EGFR-T17 cell lysates using a highly-specific anti-EGFR polyclonal antibody (Elexpuru *et al.*, 1994). The permeabilized cell system was also suitable for the assay of the EGF-dependent phosphorylation of exogenous substrates. As also shown in Figure 1 (lanes 3 and 4), the phosphorylation of the artificial substrate poly-L-(Glu, Tyr), migrating in the gels as a broad smear along the lanes, was markedly enhanced by the presence of EGF.
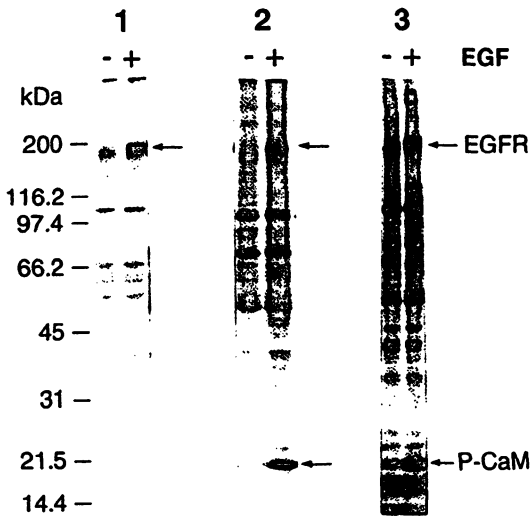
When the phosphorylation of the EGFR was assayed in the presence of different concentrations of the ligand, we obtained a half-maximal stimulation of this process at 5 nM EGF (results not shown). Phosphoamino acid analysis of the EGFR revealed that serine, threonine and tyrosine residues were phosphorylated to similar extents in the absence of EGF. Upon addition of this growth factor, however, the phosphorylation of serine and threonine residues was not significantly enhanced, whereas the phosphorylation of tyrosine residues increased by over 3-fold (results not shown).

Phosphorylation of Calmodulin by Permeabilized Cells

Since our assay system allowed the detection of EGF-dependent phosphorylation of exogenous substrates, and since the EGFR isolated from rat liver plasma membranes had been shown to phosphorylate exogenously added calmodulin (San José *et al.*, 1992; Benguría and Villalobo, 1993; Benguría *et al.*, 1994, 1995a), we tested whether EGF-dependent phosphorylation of calmodulin also occurred in permeabilized EGFR-T17 fibroblasts.

The phosphorylation of calmodulin by the rat liver EGFR had been previously shown to require the presence of a cationic polypeptide, such as poly-L-(lysine) or histones (San José *et al.*, 1992; Benguría and Villalobo, 1993; Benguría *et al.*, 1994, 1995a). Therefore, we assayed calmodulin phosphorylation by permeabilized fibroblasts in the absence and presence of these cationic polypeptides. Calmodulin was not phosphorylated in the absence of these cofactors, either in the absence or presence of EGF, whereas phosphorylated calmodulin was readily detected by including poly-L-(lysine) or histones (types H2, H3 and H4) in the assays (see Figure 2). Phosphocalmodulin displayed the Ca^{2+}-dependent electrophoretic mobility shift characteristic of non-phosphorylated calmodulin (Burgess *et al.*, 1980), migrating as a double band at 16.5 kDa (major component) in the presence of Ca^{2+} (not shown) and as a single band at 21 kDa in the presence of EGTA (Figure 2). Phosphorylation of calmodulin was stimulated 1.7 ± 0.5-fold (mean ± S.E.M., n = 3) and 2.6 ± 0.2-fold (mean ± S.E.M., n = 4) by EGF in the presence of poly-L-(lysine) and histones, respectively. In addition, the phosphorylation of a number of cellular proteins was also enhanced by these cationic polypeptides, as previously reported (Gatica *et al.*, 1987).

Optimal phosphorylation of calmodulin by distinct protein kinases, including casein kinase II (Meggio *et al.*, 1987; Sacks and McDonald, 1992), spleen tyrosine protein kinase III (Meggio *et al.*, 1987), the insulin receptor (Laurino *et al.*, 1988; Sacks and McDonald, 1988; Sacks *et al.*, 1989) and the EGFR from rat liver (San José *et al.*, 1992; Benguría *et al.*, 1994), has been reported to occur within

Fig. 1 Tyrosine Kinase Activity in Permeabilized EGFR-T17 Fibroblasts.
Permeabilized EGFR-T17 fibroblasts (2–2.5 × 10^4 cells) were incubated for 30 min at 0 °C in the absence (lanes 1 and 3) and presence (lanes 2 and 4) of 1 μM EGF, and phosphorylation was carried out at 37 °C for 3 min in the absence (lanes 1 and 2) and for 1.5 min in the presence (lanes 3 and 4) of 100 μg/ml poly-L-(Glu, Tyr) as described in Materials and Methods. The reaction was stopped by addition of ice-cold 10% (w/v) trichloroacetic acid, and the precipitated proteins were processed by SDS-PAGE and autoradiography. The arrow points to the phosphorylated EGFR in the autoradiograph, and the bracket denotes the smear corresponding to phosphorylated poly-L-(Glu, Tyr).

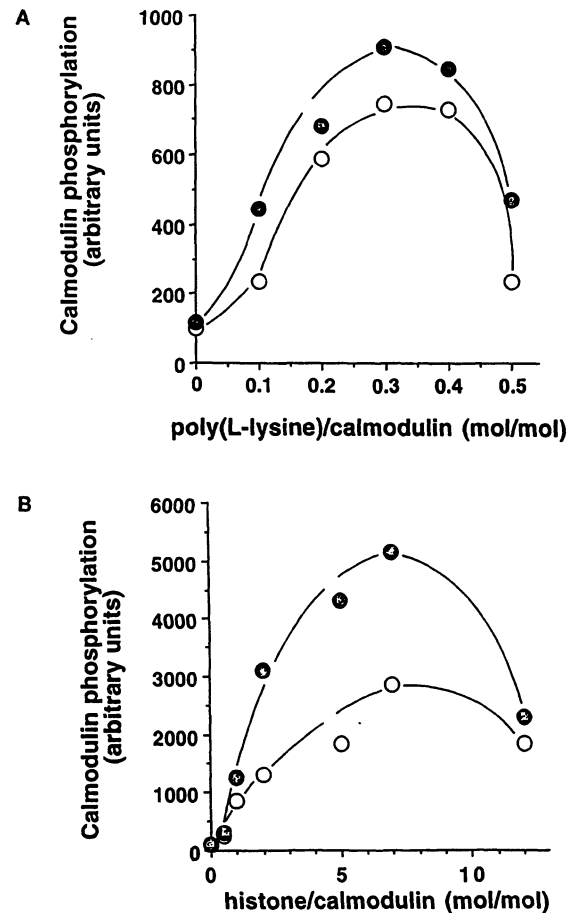**Fig. 2** Phosphorylation of Calmodulin Requires a Cationic Polypeptide.

Permeabilized EGFR-T17 fibroblasts ($2-2.5 \times 10^4$ cells) were incubated for 30 min at 0 °C in the absence (–) and presence (+) of 1 μM EGF. Thereafter, 1 μM calmodulin was added and phosphorylation assays were carried out at 37 °C for 3 min in the absence (lanes 1) and presence of either 0.3 μM poly-L-(lysine) (lanes 2) or 5 μM histones (lanes 3) as described in Materials and Methods. After stopping the reaction with ice-cold 10% (w/v) trichloroacetic acid, the precipitated proteins were dissolved in sample buffer containing 10 mM EGTA and analyzed by SDS-PAGE and autoradiography. Arrows point to the phosphorylated EGFR and to phosphorylated calmodulin (P-CaM).

narrow ranges of cationic polypeptide/calmodulin molar ratios. Above and below the optimal concentrations of cationic polypeptide, calmodulin phosphorylation was drastically reduced or undetected. In order to determine whether this effect also occurred for the human EGFR in the permeabilized-cell system, the EGF-dependent phosphorylation of calmodulin was analyzed by including different concentrations of either poly-L-(lysine) or histones while keeping constant the concentration of calmodulin.

As illustrated in Figure 3, a biphasic effect of both types of cationic polypeptides toward calmodulin phosphorylation was observed both in the absence and presence of EGF. From these experiments we calculated optimal molar ratios of approximately 0.3 for poly-L-(lysine)/calmodulin (Figure 3A) and 7 for histone/calmodulin (Figure 3B), both in the absence and presence of the ligand. These values agree well with those we have previously reported for the isolated EGFR from rat liver, of 0.2 for the poly-L-(lysine)/calmodulin molar ratio and of 3–9 for the histone/calmodulin molar ratio (Benguría et al., 1994). The one-order of magnitude lower optimal molar ratio found using poly-L-(lysine) as compared to histones is most likely attributable to the higher number of positive charges present in the former cationic polypeptide (Benguría et al., 1994).

Cationic polypeptides are known to interact with a variety of protein kinases, including the insulin receptor and

the EGF receptor, and to modify their catalytic and binding properties toward their substrates and ligands (Gatica et al., 1987; Fujita-Yamaguchi et al., 1989; Biener and Zick, 1991; Hubler et al., 1992, and references therein). Thus, it is possible that an alteration of the aggregation state of the EGFR promoted by these polypeptides could enhance its ability to phosphorylate calmodulin. Alternatively or additionally, cationic polypeptides could promote a conformational change in calmodulin facilitating its binding to these protein kinases and hence its phosphorylation, thereby mimicking the action of naturally-occurring cationic proteins (Fujita-Yamaguchi et al., 1989). In contrast, the addition of exogenous cationic polypeptides was not required for either the phosphorylation of endogenous calmodulin in vivo (Plancke and Lazarides, 1983; Fukami et al., 1985; Nakajo et al., 1986; Sacks et al., 1992; Joyal and Sacks, 1994) or the phosphorylation of membrane-bound calm-


Fig. 3 Biphasic Effect of Poly-L-(lysine) and Histones on Calmodulin Phosphorylation.

Phosphorylation of calmodulin was assayed as described in Figure 2, in the absence (open symbols) and presence (filled symbols) of 1 μM EGF, except that different concentrations of poly-L-(lysine) (panel A) or histones (panel B) were used in the assays to attain the indicated molar ratios. Phosphocalmodulin was measured densitometrically from the corresponding autoradiographs, and plotted versus the cationic polypeptide/calmodulin molar ratio. The phosphorylation of calmodulin in the absence of both EGF and cationic polypeptide was taken as 100 units.

odulin *in vitro* (Benguría *et al.*, 1995b), which suggests the existence of endogenous, positively-charged factor(s) that facilitate(s) calmodulin phosphorylation within the cell.

Calcium ions have been shown to negatively modulate the phosphorylation of calmodulin by all protein kinases studied so far, including casein kinase II (Meggio *et al.*, 1987), spleen tyrosine protein kinase III (Meggio *et al.*, 1987), rat liver and human placenta insulin receptors (Sacks and McDonald, 1988; Sacks *et al.*, 1989), rat liver plasma membrane-bound kinase(s) (Benguría *et al.*, 1995b) and the rat liver EGFR (San José *et al.*, 1992; Benguría and Villalobo, 1993; Benguría *et al.*, 1994, 1995a). In order to analyze the effect of free Ca^{2+} on calmodulin phosphorylation by the human EGFR expressed in EGFR-T17 fibroblasts, assays were carried out in the presence of varying concentrations of $CaCl_2$ to yield different concentrations of free Ca^{2+}, both in the absence and presence of EGF. As illustrated in Figure 4, maximum levels of calmodulin phosphorylation were attained in the presence of EGF and absence of Ca^{2+}. The phosphorylation of calmodulin was progressively inhibited by increasing the concentration of free Ca^{2+}, and this inhibition was very significant at $1 \mu M$ free Ca2+, as was also the case for the isolated rat liver EGFR (Benguría *et al.*, 1994). From these experiments, an apparent inhibition constant (Ki) of approximately $0.2 \mu M$ free Ca^{2+} was determined for the human EGFR, both in the absence and presence of EGF.

The inhibitory effect of Ca^{2+} has been shown not to be attributable to inhibition of the EGFR tyrosine kinase ac-
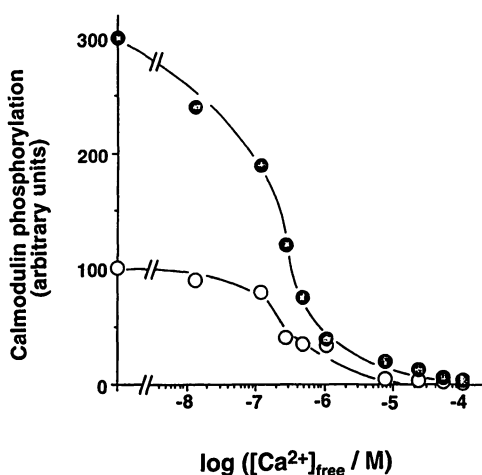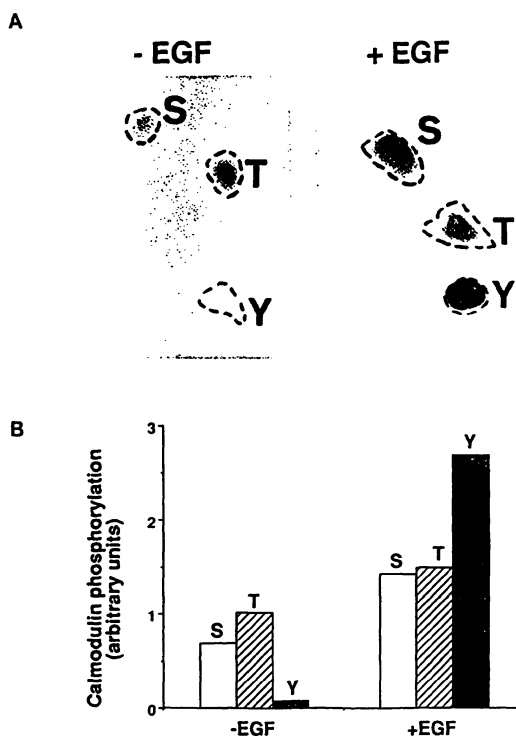

Fig. 4 Effect of Free Ca^{2+} on Calmodulin Phosphorylation. Permeabilized EGFR-T17 fibroblasts ($2-2.5 \times 10^4$ cells) were incubated for 30 min at 0 °C in the absence (open symbols) and presence (filled symbols) of $1 \mu M$ EGF. Thereafter, $1 \mu M$ calmodulin was added and phosphorylation assays were carried out at 37 °C for 3 min in the presence of $5 \mu M$ histone, $0.5 mM$ EGTA and different concentrations of $CaCl_2$ to attain the indicated concentrations of free Ca^{2+}, as described in Materials and Methods. Phosphocalmodulin levels were measured densitometrically from the corresponding autoradiograph, and plotted versus the logarithm of the concentration of free Ca^{2+}. The phosphorylation level obtained in the absence of both EGF and $CaCl_2$ was taken as 100 units.

tivity, but instead it appears to be due to a Ca^{2+}-promoted change on the conformation of calmodulin preventing its binding to the receptor and/or rendering inaccessible the phosphorylatable residue(s) (Benguría *et al.*, 1994). This contention is supported by the fact that the residue tyrosine-99, which is phosphorylated by the EGFR, is located in the third Ca^{2+}-binding site of calmodulin (Benguría *et al.*, 1994). Furthermore, the low concentration of free Ca^{2+} required to inhibit the EGFR tyrosine kinase activity suggests a physiological relevance for this modulatory process.

It has been reported using a membrane preparation from A431 human epidermoid carcinoma cells, which also overexpress the EGFR, that EGF stimulates the phosphorylation of calmodulin solely on serine residues, indicating that this phosphorylation was not directly catalyzed by the EGFR (Lin *et al.*, 1986). In order to ascertain that tyrosine was indeed phosphorylated in exogenous calmodulin using permeabilized EGFR-T17 fibroblasts, phosphoamino acid analysis of calmodulin was carried out after its phosphorylation in the absence and presence of EGF.

As illustrated in Figure 5A, EGF stimulated the phosphorylation of tyrosine and serine residues in the 21 kDa calmodulin band as excised from the gel. To quantify the actual levels of phosphoamino acids in calmodulin, control experiments without exogenous calmodulin were carried out in parallel in order to subtract the background levels of phosphoamino acids from other polypeptides possibly comigrating with calmodulin in the gels. After this correction, and as shown in Figure 5B, EGF was found to promote an increase in the phosphorylation of tyrosine residue(s) of calmodulin of approximately 34-fold, whereas serine and threonine phosphorylation increased only by 1.5- to 2-fold each as compared to the phosphorylation levels obtained in the absence of EGF. The increase in the phosphorylation of serine and threonine residues of calmodulin was due to EGF-mediated activation of serine/threonine-protein kinases.

Summarizing, permeabilized EGFR-T17 fibroblasts constitute a simple *in vitro* system to assay the EGFR tyrosine kinase activity, including both receptor transphosphorylation and phosphorylation of exogenous substrates, which avoids the need of using purified or immunoprecipitated EGFR preparations. Using this system, we have demonstrated that this receptor phoshorylates exogenous calmodulin and that this process exhibits properties similar to those reported by us for the EGFR isolated from rat liver. This suggests that EGF-stimulated phosphorylation of calmodulin could be a widespread phenomenon occurring in different cell types. The results reported in this work further support the contention that regulatory interactions probably exist between calmodulin and the EGFR (San José *et al.*, 1992; Benguría and Villalobo, 1993; Benguría *et al.*, 1994, 1995a). In addition to a possible role of calmodulin in the long-term desensitization of the EGFR that takes place upon extended exposure to its ligand (Cunningham *et al.*, 1989;

A

- EGF   **+ EGF**

B

Fig. 5 Phosphoamino Acid Analysis of Phosphocalmodulin.
(A) Permeabilized EGFR-T17 fibroblasts ($4-5 \times 10^4$ cells) were incubated for 30 min at 0 °C in the absence and presence of 1 μM EGF, as indicated. Thereafter, 1 μM calmodulin was added and phosphorylation assays were carried out at 37 °C for 3 min in the presence of 5 μM histones using 10 μM (20 μCi) [γ-^{32}P]ATP. After SDS-PAGE and autoradiography, the 21 kDa calmodulin band was excised from the dried unstained gel and phosphoamino acid analysis was performed as indicated in Materials and Methods. The positions of migration of standard phosphoserine (S), phosphothreonine (T) and phosphotyrosine (Y) stained with ninhydrin are indicated on the autoradiographs by dashed lines. (B) Graphical representation of the relative densitometric levels of the three phosphoamino acids of calmodulin phosphorylated in the absence and presence of EGF, as indicated, after subtracting the background phosphorylation levels obtained in control assays performed in the absence of added calmodulin.

Kuppuswamy and Pike, 1989; Countaway et al., 1992), calmodulin is likely to exert a short-term inhibitory role on the tyrosine kinase activity of this receptor (San José et al., 1992; Benguría and Villalobo, 1993; Benguría et al., 1994, 1995a). Both inhibitory effects of calmodulin could prevent a continued stimulation of cell proliferation by EGF. In addition, preliminary results from our laboratory suggest that calmodulin phosphorylated by the EGFR could up-regulate its tyrosine kinase activity in vitro (Benguría and Villalobo, 1993; Benguría et al., 1995a). Therefore, the balance between phosphorylated and non-phosphorylated calmodulin is likely to modulate the intrinsic catalytic activity of the EGFR in response to transient changes in the concentration of cytosolic free Ca^{2+} occurring during the EGF-induced mitogenic response. Future work should elucidate whether EGF-dependent phosphorylation of calmodulin takes place in intact cells.

Materials and Methods

Chemicals

Culture medium and FCS were purchased from Gibco-BRL. [γ-^{32}P]ATP (triethylammonium salt) (3000 Ci/mmol) and Hyperfilm-MP X-ray films were obtained from Amersham. Calmodulin (from bovine brain) was purchased from Calbiochem. Receptor-grade EGF (from mouse submaxillary glands), poly-L-(Glu, Tyr) (44.5–45.7 kDa), poly-L-(lysine) (38 kDa), calf thymus histones (type II-AS, fraction containing histones H2, H3 and H4 as observed in SDS-PAGE), ATP (sodium salt), PMSF, EGTA, leupeptin (hemisulphate), Triton X-100, TPCK-treated trypsin, phosphoserine, phosphothreonine and phosphotyrosine were products of Sigma. Hepes, SDS, ninhydrin and thin-layer (0.1 mm) cellulose-coated chromatographic plates were from Merck. Molecular mass standards for electrophoresis were purchased from Bio-Rad. Other chemicals used in this work were of analytical grade.

Cell Permeabilization and Phosphorylation Assays

Cells grown to confluency in 25 cm^2 flasks with Dulbecco's modified Eagle's medium containing 10% (v/v) FCS, 2 mM L-glutamine and 40 μg/ml gentamicin were washed with TD-buffer (137 mM NaCl, 5 mM KCl, 1 mM NaH_2PO_4, 20 mM Tris/HCl, pH 7.4) and transferred to medium without FCS. After overnight incubation, the cells were washed and scraped into TD-buffer, harvested by low-speed centrifugation and resuspended in 150 μl of TD-buffer per flask. Thereafter, 50 μl of a medium containing 60 mM Na-Hepes (pH 7.4), 24 mM $MgCl_2$, 2 mM EGTA, 0.4% (w/v) Triton X-100, 0.8 mM Na_3VO_4, 4 mM PMSF and 40 μM leupeptin was added. The cell suspension was incubated in this medium for 15 min at 0 °C and used for phosphorylation assays.

Standard phosphorylation assays were carried out for 1 to 3 min at 37 °C in 100 μl of a mixture containing 10 μl of the cell suspension (2–2.5 × 10^4 permeabilized cells), 15 mM Na-Hepes (pH 7.4), 6 mM $MgCl_2$, 0.5 mM EGTA, 0.1% (w/v) Triton X-100, 0.2 mM Na_3VO_4, 1 mM PMSF and 10 μM leupeptin. Where indicated, 100 μg/ml poly-L-(Glu, Tyr), 1 μM calmodulin, 0.3 μM poly-L-(lysine) or 5 μM histones were added. The reaction was started upon addition of 10 μM (2–3 μCi) [γ-^{32}P]ATP and stopped by addition of ice-cold 10% (w/v) trichloroacetic acid. The precipitated proteins were separated by centrifugation, the supernatants discarded and the pellets processed for electrophoresis and autoradiography as indicated below.

Other Analytical Procedures

Slab-gel electrophoresis was performed according to Laemmli (1970) at 7.5 mA overnight at room temperature in linear 5% to 20% (w/v) polyacrylamide gradient gels in the presence of 0.1% (w/v) SDS at pH 8.3. When calmodulin was present in the samples, 10 mM EGTA was included in the electrophoresis sample buffer to obtain migration of calmodulin as a single 21 kDa band. The gels were stained with Coomassie Brilliant Blue R-250 and dried under vacuum at 70 °C on Whatman 3MM Chr paper. Phosphoamino acid analysis was carried out as described (Hunter and Sefton, 1980). Briefly, the phosphorylated bands were excised from unstained dried gels and sequentially treated with 150 μg TPCK-treated trypsin for 15 h at room temperature and 6 M HCl for 2 h at 110 °C, followed by two-dimensional electrophoresis on cellulose-coated plates. X-ray films were exposed at −20 °C to obtain autoradiographs. The intensity of the radiolabeled protein bands and phosphoamino acid spots were quantified by scanning the autoradiographs in a photodensitometer. The densitometric intensities of the bands were directly proportional to the amounts of ^{32}P they contained within the exposure

times used. The concentration of free Ca^{2+} in the reaction mixtures was calculated by using a computer program similar to that described by Goldstein (1979).

Acknowledgements

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