Phosphorylation of calmodulin by the epidermal-growth-factor-receptor tyrosine kinase

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An epidermal-growth-factor(EGF)-receptor preparation isolated by calmodulin-affinity chromatography from rat liver plasma membranes is able to phosphorylate calmodulin. Calmodulin phosphorylation was enhanced 3–8-fold by EGF, was dependent on the presence of a polycation or basic protein and was inhibited by micromolar concentrations of Ca\(^{2+}\). Phosphate incorporation into calmodulin occurs predominantly on tyrosine residues. Partial proteolysis of phosphocalmodulin by thrombin identifies Tyr99, located in the third calcium-binding domain of calmodulin, as the phosphorylated residue. Stoichiometric measurements show a \(3^{32}\)P/calmodulin molar ratio of approximately 1 when optimal phosphorylation conditions are used.

Calmodulin is an intracellular calcium receptor that mediates multiple essential functions in eukaryotic cells (Means and Dedman, 1980; Klee et al., 1980; Klee and Vanaman, 1982; Manalan and Klee, 1984; Veigl et al., 1985; Strymdak and James, 1989; Bachs et al., 1992), including the regulation of cell proliferation (Veigl et al., 1984). In this context, it has been demonstrated that this ubiquitous regulator can control multiple nuclear processes (Bachs et al., 1992). Nevertheless, the role of calmodulin in cell proliferation does not appear to be exclusively exerted at the level of the nucleus. Recently we have demonstrated that the epidermal growth factor (EGF) receptor can be isolated by calmodulin-affinity chromatography, and that calmodulin inhibits its tyrosine kinase activity (San José et al., 1992). Calmodulin, therefore, could potentially play a regulatory role in the EGF-mediated mitogenic-signal pathway.

Calmodulin has been shown to be a substrate for several different protein kinases (Plancke and Lazarides, 1983; Häring et al., 1985; Fukami et al., 1985; Graves et al., 1986; Lin et al., 1986; Nakajo et al., 1986, 1988; Meggio et al., 1987, 1992; Kubo and Strott, 1988; Sacks and McDonald, 1988; Laurino et al., 1988; Sacks et al., 1989a, 1992a; San José et al., 1992; Benguria et al., 1993; Saville and Houslay, 1994). Since this phosphorylation process occurs in intact cells (Plancke and Lazarides, 1983; Fukami et al., 1985; Nakajo et al., 1986; Colca et al., 1987; Sacks et al., 1992b), phosphocalmodulin could play an important role in the physiology of the cell. In support of this, it has been shown that phosphorylation of calmodulin by casein kinase-2 (an insulin-sensitive and EGF-sensitive serine/threonine kinase) alters the biological activity of calmodulin by decreasing the activation of two calmodulin-dependent enzymes, myosin-light chain kinase and cyclic-nucleotide phosphodiesterase (Sacks et al., 1992a). Additionally, phosphorylation of calmodulin in Tyr99 by the insulin receptor decreases the action of calmodulin antagonists on the type-I cyclic-nucleotide phosphodiesterase, although it does not affect the calmodulin dependency of this enzyme (Saville and Houslay, 1994).

In a short communication Lin et al. (1986) reported that plasma membrane fractions from the human epidermal carcinoma cell line A431, which overexpress the EGF receptor, can be used to phosphorylate calmodulin in the presence of EGF. However, the authors indicated that this phosphorylation takes place on serine residues (Lin et al., 1986), suggesting that the EGF receptor is not directly involved in the phosphorylation process. We have noticed that calmodulin can be phosphorylated by an EGF-receptor preparation isolated by calmodulin-affinity chromatography (San José et al., 1992). In this study we present a comprehensive characterization of this phosphorylation process.

MATERIALS AND METHODS

Chemicals

Radioisotopic [\(\gamma^{32}\)P]ATP (triethylammonium salt) (3000–5000 Ci/mmol) was purchased from Amersham, and X-Omat AR X-ray films were purchased from Eastman Kodak. Molar-nass standards for electrophoresis were obtained from Bio-Rad, and thrombin and bovine brain calmodulin were from Calbiochem. Calmodulin-agarose, phenyl-
Sepharose, ATP (sodium salt), leupeptin, EGF (from mouse submaxillary glands), phosphotyrosine, phosphoserine, phosphothreonine, histone (type II-AS), Triton X-100, poly(l-Glu-o-Tyr-o) (44.5-45.7 kDa), and poly(l-lysine) (38 kDa) were purchased from Sigma. Thin layer (0.1 mm) cellulose-coated chromatographic plates and Hepes were obtained from Merck. Other chemicals used in this work were of analytical grade. The properties of the highly specific monoclonal antibody to calmodulin used in this study have been previously described (Sacks et al., 1991).

Preparation of liver plasma membrane fractions

Liver plasma membrane fractions from young adult male Sprague-Dawley albino rats (200-250 g) were prepared at 4°C as previously described (San José et al., 1993). In most preparations the homogenization in a glass/Teflon homogenizer was increased up to 20 strokes and the 15 s homogenization with a Polytron was omitted. To avoid proteolysis of the EGF receptor, 2 μM leupeptin was added to the buffers. For the preparation of plasma membrane fractions depleted of the calcium-dependent bound calmodulin pool, 1 mM EGTA was added to all buffers and sucrose gradient solutions. The membranes were finally resuspended in an EGTA-free buffer.

Calmodulin-affinity chromatography

The isolation of the EGF receptor by calmodulin-affinity chromatography was performed essentially as described earlier (San José et al., 1992). Briefly, membranes were solubilized in a medium containing 25 mM sodium Hapes, pH 7.4, 5% (mass/vol.) glycerol, 2 μM leupeptin and 1% (mass/vol.) Triton X-100 for 10 min at 4°C, and centrifuged at 130,000 g for 1 h. 100 μM CaCl₂ was then added to the supernatant and this was loaded onto a calmodulin-agarose column (4-5 ml bed volume containing 5-6 mg linked calmodulin) equilibrated with 25 mM sodium Hapes, pH 7.4, 5% (mass/vol.) glycerol, 1% (mass/vol.) Triton X-100 and 100 μM CaCl₂ (Ca²⁺ buffer). After extensive washing with the Ca²⁺ buffer, proteins were eluted with the same buffer containing 1 mM EGTA instead of CaCl₂, and 0.75-ml fractions were collected. It is important to solubilize the membranes in the presence of leupeptin and in the absence of exogenous Ca²⁺ to prevent partial proteolysis of the EGF receptor by Ca²⁺-dependent proteases (Wang et al., 1989). Therefore, CaCl₂ was added just before loading the column. The chromatographic procedure was carried out at 4°C.

Phosphorylation assays

Standard phosphorylation assays, unless indicated otherwise, were carried out at 37°C for 5 min in a total volume of 100 μl in a medium containing 15 mM sodium Hapes, pH 7.4, 6 mM MgCl₂, 10 μM [γ-³²P]ATP (2-5 μCi), 0.26 μM poly(l-lysine) (when added), 3-6 μM histone (when added), 1 μM calmodulin (when added), 1 μM EGF (when added) and 50 μl of the EGTA-eluted fraction from the calmodulin-agarose column. The assay system also contained 0.5 mM EGTA, 2.5% (mass/vol.) glycerol, and 0.5% (mass/vol.) Triton X-100, carried over from the EGTA-eluted fraction. In all experiments the EGTA-eluted fraction was incubated for 30 min on ice in the presence of EGF prior to phosphorylation. The reaction was initiated by adding radiolabeled ATP and stopped with ice-cold 10% (mass/vol.) trichloroacetic acid (final concentration). The precipitated proteins were separated by centrifugation, the supernatant was discarded, and the pellet was processed by electrophoresis and autoradiography as described below.

Phosphoamino acid analysis

Phosphoamino acid analysis was carried out essentially as described by Hunter and Sefton (1980). The ³²P-phosphorylated bands were cut from the dried gels, rehydrated in 100 mM NH₄HCO₃, pH 8.3, and digested in two steps with 150 μg l-tosylamido-2-phenylchloromethyl ketone (TosPh-CH₂Cl)-treated trypsin for 18 h. The supernatant was lyophilized twice and treated with 6 M HCl at 110°C for 2 h. The lyophilized samples were separated by two-dimensional electrophoresis in thin-layer (0.1 mm) chromatographic cellulose plates as follows. The first dimension was carried out in 2.2% (by vol.) formic acid and 8.7% (by vol.) acetic acid, pH 1.9, and the second dimension was carried out in 0.5% (by vol.) pyridine and 5% (by vol.) acetic acid, pH 3.5. Phosphoserine, phosphothreonine and phosphotyrosine standards were stained with 0.1% (mass/vol.) ninhydrin in ethanol. The plates were dried and autoradiography was performed.

Other analytical procedures

Slab-gel electrophoresis was performed according to Laemmli (1970) at 12 mA overnight in linear gradient from 5% to 20% (mass/vol.) polyacrylamide gels in the presence of 0.1% (mass/vol.) SDS at pH 8.3, with the addition of 10 mM CaCl₂ or 10 mM EGTA in the sample buffer. The
Fig. 2. A monoclonal antibody against calmodulin prevents its phosphorylation. Calmodulin (0.1 μM), previously incubated for 90 min at 0°C with the indicated concentrations of the monoclonal antibody against calmodulin, was phosphorylated by the EGF-receptor preparation (40 μl) in the absence and in the presence of 1 μM EGF for 3 min at 37°C in 100 μl of a medium containing 15 mM sodium Hepes, pH 7.4, 6 mM MgCl₂, 0.4% (mass/vol.) Triton X-100, 2% (mass/vol.) glycerol, 0.4 mM EGTA, 0.5 μM histone, and 100 μM [γ-32P]ATP (●). Control experiments were performed in a similar manner in the absence of calmodulin or histone, but in the presence of 0.1 mg/ml poly(L-Glu³⁰, Tyr⁵⁰) (○). The plots represent the levels of EGF-dependent phosphorylation of calmodulin (●), and of poly(L-Glu³⁰, Tyr⁵⁰) (○), as well as the EGF-dependent autophosphorylation of the EGF receptor (△).}

RESULTS

Phosphorylation of calmodulin by the EGF receptor requires the presence of a basic polypeptide

We isolated the EGF receptor from solubilized rat liver plasma membrane fractions by affinity chromatography on a calmodulin-agarose column as described earlier (San José et al., 1992). The EGTA-eluted fractions obtained from this column were used to phosphorylate calmodulin.

In the absence of a basic protein or polycation, calmodulin is not phosphorylated by the EGF receptor in the absence or presence of EGF (data not shown). Under these conditions, the major 32P-labeled protein observed in the autoradiographs is directly proportional to the amount of 32P in the bands, within the exposure time used. Protein concentrations were determined by the method of Lowry et al. (1951), after precipitating the proteins with 10% (mass/vol.) trichloroacetic acid, and using bovine serum albumin as a standard.

We also show in Fig. 1 that phosphocalmodulin exhibits the Ca²⁺-induced electrophoretic mobility shift, migrating as a double band at 16.5 kDa (major component) in the presence of calcium and as a single band at 21 kDa in the presence of EGTA. This change of mobility enables separation of phosphocalmodulin from phosphohistone in the autoradiograph. Both phosphocalmodulin (Fig. 1) and non-phosphorylated calmodulin (data not shown) migrate as two bands when electrophoresis is performed in the presence of calcium. To further ascertain that the observed 16.5-kDa/21-kDa phosphorylated band was indeed phosphocalmodulin the effect of a highly specific monoclonal antibody against calmodulin (Sacks et al. 1991) was tested on its phosphorylation. We show in Fig. 1 that phosphocalmodulin exhibits the Ca²⁺-induced electrophoretic mobility shift, migrating as a double band at 16.5 kDa (major component) in the presence of calcium and as a single band at 21 kDa in the presence of EGTA. This change of mobility enables separation of phosphocalmodulin from phosphohistone in the autoradiograph. Both phosphocalmodulin (Fig. 1) and non-phosphorylated calmodulin (data not shown) migrate as two bands when electrophoresis is performed in the presence of calcium. To further ascertain that the observed 16.5-kDa/21-kDa phosphorylated band was indeed phosphocalmodulin the effect of a highly specific monoclonal antibody against calmodulin (Sacks et al. 1991) was tested on its phosphorylation. As anticipated, EGF also enhances autophosphorylation of the EGF receptor (Fig. 1, EGFr).

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Histone is likely to account for the lower molar ratio when the phosphorylation of calmodulin increases to reach a maximum poly(L-lysine)/calmodulin molar ratio of 0.2, and an optimal poly(L-lysine) is used.

We also demonstrate that the molar ratio of poly(L-lysine) or histone to calmodulin exhibits a narrow range for the optimal phosphorylation of calmodulin. Fig. 3 presents plots of the levels of phosphorylation of calmodulin at progressively higher poly(L-lysine)/calmodulin (Fig. 3A) or histone/calmodulin (Fig. 3B) molar ratios. In both cases it is observed that the phosphorylation of calmodulin increases to reach a maximum, and dramatically decreases as these molar ratios increase. Similar results were obtained in the absence and in the presence of EGF.

Kinetic parameters for the phosphorylation of calmodulin

Some kinetic parameters for the phosphorylation of calmodulin in the presence of histone have been determined. Fig. 4A presents time-courses of phosphorylation of calmodulin carried out in the absence or presence of EGF. An initial short lag phase occurs before the reaction attains its maximum rate, then remaining virtually constant for 5 min in the presence of EGF, and for 10 min in its absence. Thereafter, the reaction rate decreases until eventually the reaction comes to a halt. The maximum reaction rate in the presence of EGF is sixfold higher than in the absence of the ligand.

When the assays are performed at different concentrations of ATP (Fig. 4B), the phosphorylation of calmodulin follows a saturation curve close to Michaelis-Menten kinetics in the presence of EGF. We calculated from this plot an apparent association constant for ATP [K_0.5(EGF)] of approximately 4–5 μM. The reaction also tends to saturation when the phosphorylation occurs in the absence of EGF. From this plot we determined that the phosphorylation of calmodulin was approximately eightfold higher in the presence than in the absence of EGF.

Phosphorylation assays were also performed at different concentrations of calmodulin (Fig. 4B), the phosphorylation of calmodulin follows a saturation curve close to Michaelis-Menten kinetics in the presence of EGF. We calculated from this plot an apparent association constant for ATP [K_0.5(EGF)] of approximately 4–5 μM. The reaction also tends to saturation when the phosphorylation occurs in the absence of EGF. From this plot we determined that the phosphorylation of calmodulin was approximately eightfold higher in the presence than in the absence of EGF.

Phosphorylation assays were also performed at different concentrations of calmodulin. The indicated concentrations of calmodulin were phosphorylated by the EGF-receptor preparation (350 μl) at 37°C for 5 min in 100 μl of a medium containing 15 mM sodium Hepes, pH 7.4, 6 mM MgCl_2, 0.5 mM EGTA, 0.5% (mass/vol.) Triton X-100, 2.5% (mass/vol.) glycerol and 50 μM [γ-32P]ATP (1.5 μCi), in the absence (Δ, ○) or presence (Δ, ●) of 1 μM EGF. The assays contained either a constant concentration (3 μM) of histone in order to vary the histone/calmodulin molar ratio (Δ, △), or variable concentrations of histone to maintain a histone/calmodulin molar ratio of 3:1 (○, ●). The plot presents the level of phosphorylation of calmodulin determined densitometrically in the autoradiograph versus the concentration of calmodulin.

We calculated from these plots an apparent association constant for calmodulin [K_0.5(Calmodulin)] of approxi-
Phosphoamino acid analysis of phosphocalmodulin. Calmodulin (1 μM) was phosphorylated by the EGF-receptor preparation (50 μl) at 37°C for 5 min in 100 μl of a medium containing 15 mM sodium Hapes, pH 7.4, 6 mM MgCl₂, 0.5 mM EGTA, 0.5% (mass/vol.) Triton X-100, 0.5% (mass/vol.) glycerol and 50 μM [γ-³²P]ATP (15 μCi), in the absence (−) or presence (+) of 1 μM EGF, and in the presence of 0.26 μM poly(l-lysine) (A), or 6 μM histone (B). The band of phosphocalmodulin was cut from the dry gel and processed for phosphoamino acid analysis as described in Materials and Methods. The position of migration of standard phosphoamino acids stained with ninhydrin are indicated in the autoradiographs by dashed lines.

Fig. 6. Phosphoamino acid analysis of phosphocalmodulin. Calmodulin (1 μM) was phosphorylated by the EGF-receptor preparation (50 μl) at 37°C for 5 min in 100 μl of a medium containing 15 mM sodium Hapes, pH 7.4, 6 mM MgCl₂, 0.5 mM EGTA, 0.5% (mass/vol.) Triton X-100, 2.5% (mass/vol.) glycerol and 50 μM [γ-³²P]ATP (15 μCi), in the absence (−) or presence (+) of 1 μM EGF, and in the presence of 0.26 μM poly(l-lysine) (A), or 6 μM histone (B). The band of phosphocalmodulin was cut from the dry gel and processed for phosphoamino acid analysis as described in Materials and Methods. The position of migration of standard phosphoamino acids stained with ninhydrin are indicated in the autoradiographs by dashed lines.

An excess of calmodulin inhibits EGF-receptor-mediated calmodulin phosphorylation (Fig. 5). This inhibitory effect is most pronounced at high concentrations of calmodulin in the absence or presence of EGF. This inhibitory action of free calmodulin confirms our earlier results (San José et al., 1992).

The EGF receptor phosphorylates calmodulin on Tyr99 with high stoichiometry

Phosphoamino acid analysis of phosphocalmodulin was performed to determine whether calmodulin is phosphorylated on tyrosine residues. Fig. 6 presents autoradiographs of the phosphoamino acids of calmodulin when the phosphorylation is performed in the absence or presence of EGF, either in the presence of poly(l-lysine) (Fig. 6A) or histone (Fig. 6B). We observe that under basal conditions (in the absence of EGF) phosphorylation of tyrosine occurs. Addition of EGF results in a significant increase of phosphorylase. Trace amounts of phosphoserine and phosphothreonine are also observed, particularly when histone is present in the assay. EGF does not increase the phosphorylation of serine or threonine residues. This indicates that the EGF receptor is not indirectly mediating the phosphorylation of calmodulin by activating serine/threonine protein kinase(s), that could be present in the EGF-receptor preparations.

Mammalian calmodulin contains two tyrosine residues, Tyr99 and Tyr138 (Klee and Vanaman, 1982). Therefore, it was of interest to determine the specific site(s) of phosphorylation. To discriminate between the phosphorylation of Tyr99 and Tyr138 we took advantage of the selective proteolysis of phosphocalmodulin by thrombin. This protease cleaves calmodulin between residues 106 and 107 yielding a major fragment containing Tyr99 and a minor fragment containing Tyr138 (Wall et al., 1981).
In Fig. 7 it is shown that bovine brain calmodulin migrates at 21 kDa on SDS/PAGE in which EGTA has been added to the electrophoresis sample buffer as observed after staining with Coomassie Blue. Treatment of non-phosphorylated calmodulin with thrombin results in the formation of a major fragment that migrates in the presence of EGTA as a polypeptide of apparent molecular mass of 14 kDa and a minor fragment of lower molecular mass, hardly visible in the gel. Thrombin is observed in Fig. 7 lane 2 as a 35-kDa band. Fig. 7 also presents the autoradiograph of 32P-labeled calmodulin isolated by phenyl-Sepharose chromatography and its 32P-labeled fragment. Calmodulin was phosphorylated by the isolated EGF receptor in the absence or presence of EGF, using [γ-32P]ATP. Lanes 3 and 4 (Fig. 7) present the autoradiograph of non-treated phosphocalmodulin, while lanes 5 and 6 (Fig. 7) present samples after treatment with thrombin. All the 32P is incorporated into the higher-molecular-mass calmodulin fragment of 14 kDa, containing Tyr99, both in the absence and presence of EGF.

We have shown that phosphorylation of calmodulin by the EGF receptor tyrosine kinase occurs in the absence of Ca2+ (see above), and we have previously noticed that Ca2+ efficiently prevents this phosphorylation (San José et al., 1992). Nevertheless, the concentrations of Ca2+ previously used were well above a concentration range of physiological relevance. Therefore, we decided to analyze the effect of lower concentrations of this cation on the phosphorylation of calmodulin. Fig. 8 shows that 1 μM free Ca2+ efficiently inhibits the phosphorylation of 1 μM calmodulin. This inhibitory effect was observed in the absence or in the presence of EGFR. Inhibition of calmodulin phosphorylation occurs when both histone and poly(L-lysine) were used as cofactors, although the inhibitory effect was more pronounced in the presence of the former.

The stoichiometry of calmodulin phosphorylation at different concentrations of calmodulin was also examined. The plot presented in Fig. 9 shows the different experimental stoichiometric ratios determined in the absence or presence of EGF. In the absence of EGF, the stoichiometry was low, with values between 0.1 mol phosphate/mol calmodulin and 0.26 mol phosphate/mol calmodulin at concentrations of calmodulin of 850 nM and 85 nM, respectively. Addition of EGF significantly increases the stoichiometry to 0.61 mol/mol and 1.04 mol/mol at high and low concentrations of calmodulin, respectively. To determine the maximum mechanistic stoichiometry we extrapolated the straight line formed by the values of experimental stoichiometries using a linear regression to a point where the concentration of calmodulin tends to zero. Under these conditions, a theoretical stoichiometry of 0.3 was determined in the absence of EGF, and of 1.1 in its presence.

**DISCUSSION**

The EGF-receptor preparation used for the phosphorylation of calmodulin in this work was isolated by calmodulin-affinity chromatography (San José et al., 1992). It is known that the insulin receptor can also be isolated by this procedure (Graves et al., 1985), and we have observed that our preparations contain the insulin receptor (Benguría et al., 1993). However, autophosphorylation of the β subunit of the insulin receptor was undetectable in the absence of insulin, and was observed only in the presence of 50 nM insulin (Benguría et al., 1993). Furthermore, no insulin was present in our assays. Our results demonstrate that EGF markedly enhances the phosphorylation of both calmodulin and the EGF receptor. Additionally, the kinetic parameters of calmodulin phosphorylation, and the requirements for a basic protein or polycation were identical in the absence or presence of EGF. The only difference observed was in the rate of phosphorylation. Furthermore, both the EGF-receptor autophosphorylation and the kinase activity toward calmodulin, are equally enriched in the isolated EGF-receptor preparation by a similar factor (14-fold and 20-fold, respectively) when compared to the plasma membranes (data not shown). Therefore, it is extremely unlikely that the insulin receptor or other tyrosine kinase was responsible for significant phosphorylation.
tion of calmodulin under the experimental conditions employed in this work.

It has been reported that poly(t-lysine) activates the EGF-receptor tyrosine kinase (Hubler et al., 1992). Therefore, poly(t-lysine) is expected to enhance the phosphorylation of calmodulin by this mechanism. This is supported by 1H-NMR and sedimentation equilibrium centrifugation studies showing that poly(t-lysine) associates with calmodulin in aqueous solution (Hardy et al., 1994).

We have demonstrated here that the EGF receptor phosphorylates calmodulin on Tyr99. Nevertheless, the poor resolution of the low-molecular-mass thrombolytic fragment of calmodulin in the gels prevents us from excluding the possibility that Tyr138 is not also phosphorylated to some extent. Tyr99 is also phosphorylated in calmodulin by the insulin receptor (Laurino et al., 1988). Additionally, Tyr99, and to a lesser extent Tyr138, are both phosphorylated by the spleen tyrosine kinase type III (Meggio et al., 1987). There appears to be a striking difference in electrophoretic mobility between the species of phosphocalmodulin yielded upon phosphorylation with the insulin receptor and the EGF receptor. Non-phosphorylated calmodulin exhibits an altered migration on SDS/PAGE in the presence of calcium compared to its mobility in the absence of calcium (Burgess et al., 1980). A similar shift is observed with calmodulin phosphorylated by the EGF-receptor tyrosine kinase (San José et al., 1992; this work). In contrast, it has been reported that the electrophoretic migration of calmodulin phosphorylated by the insulin-receptor tyrosine kinase is not changed (Laurino et al., 1988), or only partially changed (Saville and Houslay, 1994) in the presence versus the absence of calcium. The reason(s) for these discrepancies are not clear but methodological differences might account for the disparities.

The concentration of cytoplasmic free Ca\(^{2+}\) is \(10^{-8} - 10^{-7}\) M in quiescent non-stimulated cells (Carafoli, 1987), and an early event occurring in cells activated by EGFR and several other growth factors is a transient rise in the concentration of cytoplasmic free calcium reaching values of \(10^{-6} - 10^{-5}\) M (Rozengurt, 1986; Moo Lenaar et al., 1986; Pandiella et al., 1988). Calcium mobilization has been shown to be mediated by two concurrent mechanisms, although the relative importance of these two systems varies among cell types: the EGF-receptor tyrosine kinase activates phospholipase C\(y\) which hydrolyzes phosphatidylinositol lipids, resulting in the release of inositol triphosphate (Nishibe et al., 1990) that in turn activates intracellular Ca\(^{2+}\)-channels most probably located in the endoplasmic reticulum (Berridge, 1993); and the sequential activation of extracellular voltage-insensitive and voltage-sensitive Ca\(^{2+}\) channels in the plasma membrane by leukotriene C\(4\), formed by a phospholipase A\(_2/5\)-lipxygenase pathway. This pathway is thought to be activated by phosphorylation of phospholipase A\(_2\) by the EGF-receptor tyrosine kinase (Peppelenbosch et al., 1992). We have demonstrated that phosphorylation of calmodulin by the EGF receptor is very sensitive to low concentrations of Ca\(^{2+}\). Free cytosolic Ca\(^{2+}\) undergoes oscillations (Berridge, 1990) which should modulate the extent of calmodulin phosphorylation. Therefore, it is reasonable to hypothesize that the levels of phosphorylation of calmodulin may exhibit analogous fluctuations in vivo.

Phosphorylation of calmodulin in vitro by the EGF-receptor tyrosine kinase only takes place in the presence of a basic protein or polycation. This has been previously observed when calmodulin is phosphorylated in vitro by the insulin-receptor tyrosine kinase (Graves et al., 1986; Sacks and McDonald, 1988; Sacks et al., 1989a; Laurino et al., 1988), by casein kinase-2 (Meggio et al., 1992; Sacks and McDonald, 1992), and by soluble spleen tyrosine kinase type III (Meggio et al., 1987). Since insulin enhances calmodulin phosphorylation in intact cells without the addition of a cationic protein or polypeptide (Sacks et al., 1992b), an endogenous basic protein may be implicated in the phosphorylation of calmodulin in vivo. The nature of this endogenous cofactor is of considerable interest as its identification could aid in the understanding of this phosphorylation process. The highly basic C-terminal region of the protein product of the c-Ki-ras protooncogene has been implicated in the phosphorylation of calmodulin by the insulin receptor (Sacks et al., 1989b; Fujita-Yamaguchi et al., 1989). This observation could also be of great relevance to the mechanism of calmodulin phosphorylation by the EGF-receptor tyrosine kinase. The Ras protein superfamily is central to the mechanism of cell proliferation and differentiation (Huangbruck and McCormick, 1991), and the putative implication of the c-Ki-ras protein product in the control of the activity of the EGF receptor via calmodulin, could underscore a possible new mechanism for the control of mitosis by this protooncogene.

Since calmodulin is involved in the control of a great number of cellular processes (Means and Dedman, 1980; Klee et al., 1980; Klee and Vanaman, 1982; Manalan and Klee, 1984; Veigel et al., 1984; Strynadka and James, 1989; Bachs et al., 1992), we can anticipate that EGF-receptor-Tyr99-phosphorylated calmodulin may have profound effects on the different calmodulin-dependent pathways.

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