Tyrosine-specific phosphorylation of calmodulin by the insulin receptor kinase purified from human placenta

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It has previously been demonstrated that calmodulin can be phosphorylated *in vitro* and *in vivo* by both tyrosine-specific and serine/threonine protein kinase. We demonstrate here that the insulin receptor tyrosine kinase purified from human placenta phosphorylates calmodulin. The highly purified receptors (prepared by insulin–Sephacrose chromatography) were 5–10 times more effective in catalysing the phosphorylation of calmodulin than an equal number of partially purified receptors (prepared by wheat-germ agglutinin–Sephacrose chromatography). Phosphorylation occurred exclusively on tyrosine residues, up to a maximum of 1 mol $[0.90 \pm 0.14$ ($n = 5$)] of phosphate incorporated/mol of calmodulin. Phosphorylation of calmodulin was dependent on the presence of certain basic proteins and divalent cations. Some of these basic proteins, i.e. polylysine, polyarginine, polyornithine, protamine sulphate and histones H1 and H2B, were also able to stimulate the phosphorylation of calmodulin via an insulin-independent activation of the receptor tyrosine kinase. Addition of insulin further increased incorporation of ^{32}P into calmodulin. The magnitude of the effect of insulin was dependent on the concentration and type of basic protein used, ranging from 0.5- to 9.0-fold stimulation. Maximal phosphorylation of calmodulin was obtained at an insulin concentration of 10^{-10} M, with half-maximal effect at 10^{-11} M. Either Mg^{2+} or Mn^{2+} was necessary to obtain phosphorylation, but Mg^{2+} was far more effective than Mn^{2+} . In contrast, maximal phosphorylation of calmodulin was observed in the absence of Ca^{2+} . Inhibition of phosphorylation was observed as free Ca^{2+} concentration exceeded $0.1 \mu\text{M}$, with almost complete inhibition at $30 \mu\text{M}$ free Ca^{2+} . The K_m for calmodulin was approx. $0.1 \mu\text{M}$. To gain further insight into the effects of basic proteins in this system, we examined the binding of calmodulin to the insulin receptor and the polylysine. Calmodulin binds to the insulin receptor in a Ca^{2+} -dependent manner, whereas it binds to polylysine seemingly by electrostatic interactions. These studies identify calmodulin as a substrate for the highly purified insulin receptor tyrosine kinase of human placenta. They also demonstrate that the basic proteins, which are required for insulin to stimulate the phosphorylation of calmodulin, do so by a direct interaction with calmodulin.

INTRODUCTION

The insulin receptor is a transmembrane glycoprotein consisting of two α -subunits (135 kDa) and two β -subunits (95 kDa), linked together by disulphide bonds [1]. Binding of insulin to the extracellular domain in the α -subunit results in activation of tyrosine kinase, which is intrinsic to the β -subunit. Autophosphorylation of the receptor has been observed in a variety of intact cells [2], and in a large number of preparations of partially purified receptors, including those obtained from placenta, hepatocytes, 3T3-L1 adipocytes, lymphocytes and erythrocytes (for review, see [3]). In addition to the insulin receptor, receptors for a number of growth factors, e.g. epidermal growth factor [4], insulin-like growth factor I [5], and platelet-derived growth factor [6], as well as for several oncogene products [7], contain tyrosine kinase. Although tyrosine kinase activity appears to be involved in the intracellular signal transduction of these hormones and growth factors, the precise mechanism of involvement is unknown.

A number of intracellular substrates for the insulin

receptor tyrosine kinase have been identified in the last few years. These include a 110–120 kDa protein in cell-free preparations of rat liver [8,9], a 185 kDa protein in Fao hepatoma cells [10], a 15 kDa protein in 3T3-L1 adipocytes [11] and three proteins of 116 kDa, 62 kDa and 46 kDa in adipocytes [12]. Despite the identification of these phosphoproteins, their functional significance remains unknown.

Phosphorylation of calmodulin has been observed in intact cells [13,14], and a number of protein kinases have been shown to phosphorylate calmodulin *in vitro*. Among these are phosphorylase kinase [13], *src* kinase [15], casein kinase II and a spleen tyrosine kinase [16]. In addition, calmodulin can be phosphorylated by the insulin receptor tyrosine kinase both in the intact cell [17] and in some isolated receptor preparations [18–21]. We have previously observed, with insulin receptors partially purified by wheat-germ agglutinin (WGA)–Sephacrose chromatography from rat adipocytes [19], hepatocytes [20] and skeletal muscle [21], that insulin stimulates the phosphorylation of calmodulin. The phosphorylation of calmodulin has not been investigated with human tissue

Abbreviations used: WGA, wheat-germ agglutinin; BAEE, N^{α} -benzoyl-L-arginine ethyl ester; PBS, phosphate-buffered saline; PAGE, polyacrylamide-gel electrophoresis.

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or a highly purified insulin receptor kinase preparation. We have therefore investigated the ability of the tyrosine kinase of insulin receptors purified from human placenta to phosphorylate calmodulin. During the course of these studies, we found that certain basic proteins, such as polylysine or protamine sulphate, are necessary in the assay to obtain insulin-stimulated phosphorylation of calmodulin. These proteins themselves activate the kinase independently of insulin, an effect which is further characterized in the next paper [22].

EXPERIMENTAL

Materials

Protamine sulphate, poly(L-lysine) (30 000–70 000 kDa; mean 41 000 kDa), poly(L-arginine) (15 000–70 000 kDa; mean 40 000 kDa), poly(L-ornithine) (15 000–30 000 kDa; mean 25 000 kDa), spermine, spermidine, histone H1, putrescine, phenylmethylsulphonyl fluoride, *O*-phospho-L-tyrosine, *O*-phospho-L-serine, *O*-phospho-L-threonine, poly(L-lysine)-Sepharose and Sepharose CL-6B were obtained from Sigma (St. Louis, MO, U.S.A.). The following reagents were obtained from the manufacturers listed: histone H2B from Worthington Biochemical (Freehold, NJ, U.S.A.); Protosol and Aquasol 2 from Du Pont–New England Nuclear (Boston, MA, U.S.A.); 3a70 liquid scintillation mixture from Research Products International Corp. (Mt. Prospect, IL, U.S.A.); Ca^{2+} -free pig brain calmodulin from Ocean Biologics, Inc. (Edmonds, WA, U.S.A.); cyanogen bromide-activated Sepharose 4B from Pharmacia (Piscataway, NJ, U.S.A.); iodo-beads and disuccinimidyl suberate from Pierce (Rockford, IL, U.S.A.); [γ - ^{32}P]ATP ($> 2000 \text{ Ci/nmol}$) and ^{125}I (16.4 mCi/ μg) from Amersham (Arlington Heights, IL, U.S.A.). All reagents for polyacrylamide-gel electrophoresis (PAGE) and P-6DG desalting gel were purchased from Bio-Rad (Richmond, CA, U.S.A.). Partisil 10-SAX h.p.l.c columns were from Whatman (Clifton, NJ, U.S.A.).

Purification of insulin receptors

Insulin receptors were purified by sequential affinity chromatography on WGA- and insulin-Sepharose as previously described [23]. The eluate from the WGA-Sepharose column represents the partially purified receptor preparation. Fractions with insulin-binding activity were further purified on an insulin-Sepharose column. This preparation, termed the purified receptor preparation, has previously been characterized [23,24] and represents an approx. 300-fold purification of the WGA-Sepharose eluate.

Protein phosphorylation assays

Unless otherwise indicated, purified placental insulin receptors (0.5–5 μg ; approx. 0.03–0.3 μg) or receptors partially purified by WGA-Sepharose affinity chromatography (50 μg) were preincubated (in a final volume of 250 μl) in 50 mM-Tris/HCl (pH 7.5), 0.1 % (v/v) Triton X-100, 1 mM-EGTA, 0.4 mM- CaCl_2 , $6.7 \times 10^{-8} \text{ M}$ -insulin in 0.01 % bovine serum albumin, 1.2 μM -calmodulin and 0.24 μM -polylysine or the concentrations of protamine sulphate indicated in the Figure legends, for 30 min at 22 °C. The reaction was initiated by the addition of 5 mM- MgCl_2 and 50 μM -[γ - ^{32}P]ATP (10–20 μCi). After incubation for 30 min at 30 °C, the reaction was stopped by the addition of 250 μl of 10 % (w/v) trichloroacetic

acid at 4 °C. Samples were centrifuged at 37 000 g for 30 min at 4 °C, the supernatant was aspirated and solubilization buffer containing 20 mM-Tris/HCl (pH 7.5), 2 % (w/v) SDS, 2 % (v/v) β -mercaptoethanol, 0.01 % (w/v) Bromophenol Blue, 0.25 M-sucrose and 2 mM-EDTA was added to the pellets. The samples were heated at 100 °C for 5 min and applied to the gels.

Proteins were separated by SDS/PAGE in 10 % (w/v) polyacrylamide gels with a 6 % (w/v) acrylamide stacking gel. The gels were dried on filter paper and autoradiography was performed. Incorporation of ^{32}P into calmodulin was quantified. The calmodulin band was cut out of the gel, solubilized in water/Protosol/Aquasol (1:2:10, by vol) and radioactivity was measured. Background radioactivity was determined by cutting out a similarly sized portion of the gel which did not contain specifically enhanced radioactivity, and processing as described above. The 17 kDa protein was previously proved to be calmodulin [19].

Placental receptor binding to calmodulin

Calmodulin-Sepharose was prepared according to the method of Niggli *et al.* [25], with the following modifications. Both incubations were performed at 4 °C for 18 h, and 0.2 M-glycine was substituted for 0.5 M-aminoethanol. The final washes were performed with 1 M-NaCl in 0.1 M-boric acid (pH 8.0) alternating with 1 M-NaCl in 0.1 M-sodium acetate (pH 4.0). Calmodulin-Sepharose (3 ml) was equilibrated with 10 column vol. of 50 mM-Tris/HCl (pH 7.5) in a 1 cm $\times$ 6 cm column. Phosphorylated purified placental receptor preparation (5 μl ; approx. 0.3 μg) was added to the column in the presence of 0.1 μM -free Ca^{2+} , $6.7 \times 10^{-8} \text{ M}$ insulin and 0.01 % (w/v) bovine serum albumin, and then washed with 10 column vol. of equilibration buffer containing Ca^{2+} . Elution was performed with 10 mM-EGTA (pH 7.5). Respective fractions were collected from each wash, pooled and lyophilized. PAGE, autoradiography and quantification were performed as described above.

Binding of calmodulin to polylysine-Sepharose

^{125}I -Calmodulin was prepared by preincubating two iodo-beads in 400 μl of 200 mM-Tris/HCl (pH 7.5) containing 0.5 mM- CaCl_2 for 5 min at 22 °C. Calmodulin (100 μg) was added, allowed to react for 2 min at 22 °C and the reaction was stopped by applying 250 μl of sample to a P-6DG column (0.8 cm $\times$ 20 cm) that had been equilibrated with 20 ml of phosphate-buffered saline (PBS; 50 mM-sodium phosphate/150 mM-NaCl, pH 7.5). Non-specific binding to the column was prevented by adding 2 ml of 1 % (w/v) bovine serum albumin in PBS before adding the sample. Calmodulin was eluted with PBS and 2 ml fractions were collected. ^{125}I -Calmodulin was used only if trichloroacetic acid-precipitable counts exceeded 90 % of the total counts, and it bound to adipocyte membranes in a Ca^{2+} -dependent manner [26].

A portion (50 μl) of polylysine-Sepharose (0.5–1.0 mg of polylysine/ml of packed gel) was washed with 1 ml of 0.5 M-Tris/HCl (pH 7.5) and then incubated with 2 μl of ^{125}I -calmodulin for 10 min at 22 °C. Additions were made as indicated in the Figure legends and the reaction was allowed to continue for 10 min at 22 °C. After washing with 15 ml of 0.5 M-Tris/HCl (pH 7.5), the radioactivity bound to the polylysine-Sepharose was determined. Sepharose which had no polylysine attached was used as a control. The polylysine-Sepharose used

Fig. 1. The effect of insulin, polylysine and protamine sulphate on the phosphorylation of calmodulin and the β -subunit of the insulin receptor

(a) Receptors partially purified by WGA–Sepharose chromatography, (b) receptors purified by insulin–Sepharose chromatography. Phosphorylation assays were performed as described in the Experimental section in the presence (+) or absence (–) of insulin and in the presence of $0.24\ \mu\text{M}$ -polylysine or $17\ \mu\text{M}$ -protamine sulphate (as indicated). A representative autoradiogram is shown. The positions of migration of the β -subunit of the insulin receptor (β) and of calmodulin (CaM) are shown.

in the binding studies was capable of supporting phosphorylation of calmodulin when used instead of free polylysine in our standard phosphorylation assay (results not shown).

Analysis of phosphoamino acids in calmodulin

Phosphorylated calmodulin was located by autoradiography, excised from the gel, digested in trypsin and hydrolysed in 6 M-HCl as described [20]. Phosphoamino acids were separated by h.p.l.c. and quantified by liquid scintillation spectrometry as described previously [20].

Other methods

Protein content was determined by the method of Lowry *et al.* [27]. Free Ca^{2+} in the assay mixtures was measured using an Orion Research Inc. Ca^{2+} electrode 93-20, reference electrode 90-01 and microprocessor pH/millivoltmeter 811 [28].

RESULTS

Phosphorylation of calmodulin and the β -subunit of the insulin receptor using partially purified and purified placental insulin receptors

Phosphorylation of calmodulin was observed in assays utilizing insulin receptors from human placenta which were partially purified by WGA–Sepharose affinity chromatography (Fig. 1a). This preparation is comparable with the partially purified, solubilized insulin receptor from rat adipocytes [19], liver [20] and skeletal muscle [21] that we have previously studied. As was previously noted in these other preparations, there is an absolute requirement for the presence of certain basic proteins to obtain insulin-stimulated phosphorylation of calmodulin. Although we previously reported that polylysine alone moderately stimulates hepatocyte insulin

receptor kinase [20], in the placental receptor system both polylysine and protamine sulphate alone markedly stimulated phosphorylation of calmodulin in the absence of insulin (Fig. 1a, lanes 4 and 6). Nevertheless, addition of insulin to the assay, irrespective of the cofactor used, increased incorporation of ^{32}P into calmodulin by approx. 2-fold (Fig. 1a, lanes 3 and 5).

Phosphorylation of the β -subunit of the insulin receptor followed the same general pattern as the phosphorylation of calmodulin. It was stimulated by either polylysine or protamine sulphate alone (Fig. 1a, lanes 4 and 6), but addition of insulin considerably enhanced phosphorylation (lanes 3 and 5). The insulin-independent activation of the insulin receptor kinase and phosphorylation of the receptor by various basic proteins is investigated in depth in the next paper [22]. The extent of autophosphorylation of the receptor β -subunit appears to correlate with the amount of ^{32}P incorporated into calmodulin, except where protamine sulphate and insulin are added together (Fig. 1a, lane 5) in which case the β -subunit is markedly phosphorylated compared with the other conditions. The reason for this is unknown, but may be due to activated serine/threonine kinases in this preparation. In addition, when protamine sulphate is included (Fig. 1a, lanes 5 and 6), there is moderate phosphorylation of protamine sulphate itself (the bands below calmodulin) which also appears to be partially insulin-sensitive. This is probably also due to activation of serine/threonine kinase, since the salmon protamines used here contain no tyrosine residues, but are known to be good substrates for serine/threonine kinase such as protein kinase C [29].

The effects of insulin on the phosphorylation of calmodulin by the partially (Fig. 1a) and highly (Fig. 1b) purified receptor preparations were compared under the same conditions using the same number of insulin receptors per assay. The two cofactors produced similar

Fig. 2. Insulin-dependence of the phosphorylation of calmodulin

Phosphorylation assays were performed as described in the Experimental section in the presence of 0.8 mM-CaCl₂, 5 μ M-protamine sulphate and various concentrations of insulin as indicated. Incorporation of ³²P into calmodulin (CaM) was quantified and is expressed as a percentage of the maximum, which was obtained variably between 10⁻¹⁰ M- and 10⁻⁹M-insulin. Each point represents the mean of four separate experimental determinations $\pm$ S.E.M.

qualitative responses in the partially purified and highly purified receptor preparations (Figs. 1a and 1b), but purified receptors are 5–10-fold more active in phosphorylating calmodulin than partially purified receptors. This may indicate that inhibitors or phosphatases that could affect the degree of calmodulin phosphorylation were removed during purification. However, with partially purified receptors, the addition of the phosphatase inhibitors vanadate and NaF did not increase the phosphorylation of calmodulin. In both Fig. 1(a) and Fig. 1(b), longer exposure of the autoradiographs clearly shows that in lane 1 (insulin present), but not in lane 2 (no insulin present), the β -subunit of the receptor is phosphorylated. Calmodulin is phosphorylated more than the β -subunit because it is present at a higher concentration and assay conditions are optimized for phosphorylation of calmodulin. Of note is the fact that protamine sulphate is not phosphorylated (indicating that serine/threonine kinases have been removed) and β -subunit phosphorylation is not markedly stimulated by protamine sulphate plus insulin (lane 5) when a highly purified receptor is used. These highly purified receptors were utilized in all subsequent studies.

Insulin-concentration dependence of the phosphorylation of calmodulin

Fig. 2 illustrates the effect of varying insulin concentrations on phosphorylation of calmodulin. Phosphorylation of calmodulin occurred in the absence of insulin, but addition of insulin increased incorporation of ³²P into calmodulin in a concentration-dependent manner up to maximum stimulation which averaged 1.5-fold. Maximal phosphorylation was obtained at approx. 10⁻¹⁰ M-insulin, with half-maximal effect at 10⁻¹¹ M. An unexpected decrease in the insulin effect was observed at insulin concentrations above 5 $\times$ 10⁻⁹ M-insulin and at

Fig. 3. Mg²⁺- and Mn²⁺-dependence of phosphorylation of calmodulin

Phosphorylation assays were carried out as described in the Experimental section in the presence of 6.7 $\times$ 10⁻⁸ M-insulin and 0.24 μ M-polylysine, using the concentrations of MgCl₂ or MnCl₂ indicated. A representative autoradiogram is shown. The position of migration of calmodulin (CaM) is indicated.

10⁻⁷ M-insulin the stimulatory effect was essentially abolished.

Effect of Mg²⁺ and Mn²⁺ on phosphorylation of calmodulin

Phosphorylation of calmodulin was maximal in the presence of Mg²⁺ (Fig. 3). In the absence of both Mg²⁺ and Mn²⁺, no phosphorylation of calmodulin was observed (results not shown). When Mn²⁺ was substituted for Mg²⁺, there was minimal phosphorylation (Fig. 3, lanes 2, 4, 6 and 8). In contrast, Mg²⁺ supports phosphorylation in a concentration-dependent manner with 10 mM being maximum (lanes 1, 3, 5 and 7). At 20 mM-Mg²⁺, the phosphorylation of calmodulin was approx. 50% of that obtained at 10 mM-Mg²⁺ (results not shown). Furthermore, addition of both Mg²⁺ and Mn²⁺ together produced ³²P incorporation that was less than that obtained in the presence of Mg²⁺ alone, but more than was observed in the presence of Mn²⁺ alone (results not shown). Mg²⁺ may be more effective than Mn²⁺ in our assay system for a number of reasons. (i) Mn²⁺ has a high affinity constant for EGTA (10^{12.3}), 25 times greater than that of Ca²⁺ for EGTA [30]. In the presence of Mn²⁺, Ca²⁺ is displaced from EGTA resulting in high free [Ca²⁺] which inhibits phosphorylation of calmodulin (see below). The affinity constant of Mg²⁺-EGTA, however, is more than five orders of magnitude lower than the affinity constant for Ca²⁺-EGTA [30], and Mg²⁺ will therefore not displace Ca²⁺ from EGTA. (ii) There may be an important interaction between Mg²⁺ and calmodulin. Calmodulin contains four Mg²⁺-binding sites, which are separate from the Ca²⁺-binding sites [31]. The Ca²⁺- and Mg²⁺-binding sites influence each other's affinity due to negative free-energy coupling between the sites [31]. Calmodulin also has common binding sites for both Ca²⁺ and Mg²⁺ [32]. These observations suggest that Mg²⁺ may affect the

Fig. 4. Ca^{2+} -dependence of the phosphorylation of calmodulin

Phosphorylation assays were performed as described in the Experimental section in the presence of insulin and $5\text{ }\mu\text{M}$ -protamine sulphate, or $0.24\text{ }\mu\text{M}$ -polylysine, 1 mM -EGTA and varying CaCl_2 concentrations to yield the Ca^{2+} :EGTA ratios indicated. Incorporation of ^{32}P into calmodulin is expressed as a percentage of incorporation obtained in the absence of Ca^{2+} (EGTA alone). Each point represents the mean of three separate experimental determinations $\pm$ S.E.M., except for Ca^{2+} :EGTA ratios of 0.8 and 1.0 which were performed six times, 1.2 which was performed five times and 0.975 which was performed twice. The free Ca^{2+} concentrations corresponding to the Ca^{2+} :EGTA ratios of 0.8, 1.0 and 1.2 are 0.1, 30 and $200\text{ }\mu\text{M}$ respectively. Ca^{2+} :EGTA ratios of less than 0.8 have free Ca^{2+} concentrations of less than $0.08\text{ }\mu\text{M}$. A representative autoradiogram is also shown. The position of migration of calmodulin (CaM) is indicated.

phosphorylation of calmodulin. (iii) There may be an effect of Mg^{2+} on the interactions between polylysine, calmodulin and the insulin receptor [33,34].

Ca^{2+} -concentration dependence of the phosphorylation of calmodulin

Phosphorylation of calmodulin was observed in the absence of Ca^{2+} (EGTA alone) (Fig. 4). Addition of submicromolar concentrations of free Ca^{2+} to the assay produced no effect. Increasing the Ca^{2+} :EGTA ratio above 0.8 (free $[\text{Ca}^{2+}]$ of $0.1\text{ }\mu\text{M}$ which is the approximate resting intracellular Ca^{2+} concentration) resulted in inhibition of phosphorylation of calmodulin, with almost complete inhibition at Ca^{2+} :EGTA of 1.0. Although the error bars are fairly large for some of the intermediate points, there is clearly marked inhibition at the Ca^{2+} :EGTA ratios of 1.0 and 1.2 (free $[\text{Ca}^{2+}]$ of $30\text{ }\mu\text{M}$ and $200\text{ }\mu\text{M}$ respectively). Addition of 1 mM - Ca^{2+} without EGTA results in virtually complete inhibition of calmodulin phosphorylation (results not shown).

Effect of basic proteins and insulin on phosphorylation of calmodulin

In all insulin-receptor systems tested to date, calmodulin phosphorylation was dependent on the presence of certain basic proteins [19–21] (Figs. 1a and 1b). With purified placental receptors, however, the insulin-independent effects of the basic proteins such as polylysine

Fig. 5. Effect of basic proteins and insulin on phosphorylation of calmodulin

Phosphorylation assays were performed as described in the Experimental section in the presence of $1\text{ }\mu\text{M}$ of the basic proteins indicated, in the presence (hatched bars) or absence (open bars) of $6.7 \times 10^{-9}\text{ M}$ -insulin, as indicated. Incorporation of ^{32}P into calmodulin (CaM) was quantified and is expressed as a percentage of the incorporation in the presence of $1\text{ }\mu\text{M}$ -polylysine plus insulin.

and protamine sulphate on the phosphorylation of calmodulin were considerably greater than observed with partially purified receptors from adipocytes, hepatocytes or skeletal muscle. We therefore examined the relative effects of a number of basic proteins (at a concentration of $1\text{ }\mu\text{M}$), in both the presence and the absence of insulin, on phosphorylation of calmodulin using purified human placental receptors (Fig. 5). Polylysine, polyarginine, polyornithine, protamine sulphate and histones H1 and H2B all proved effective in mediating the phosphorylation of calmodulin, independently of insulin. In addition, each of these basic proteins supports insulin-stimulated calmodulin phosphorylation. The relative insulin-induced stimulation, expressed as a ratio of the value obtained in the presence of insulin divided by that obtained in its absence, is greater in the presence of histone H1 (9-fold) and least in the presence of polylysine (1.5-fold). In contrast, the magnitude of the stimulation by insulin (i.e. the value obtained in the presence of insulin minus that obtained in its absence) is greatest with polyarginine and polylysine. When spermine, spermidine or putrescine were present in the assay, no incorporation of ^{32}P into calmodulin could be obtained (results not shown). The effectiveness of the basic proteins in supporting both basal and insulin-stimulated phosphorylation of calmodulin was concentration-dependent. For example, stimulation of phosphorylation at $17\text{ }\mu\text{M}$ -protamine sulphate (Fig. 1b, lanes 5 and 6) was more than 30 times greater than stimulation observed at $1\text{ }\mu\text{M}$ -protamine sulphate. The stimulation produced by insulin was also dependent on the concentration of the cofactor (see below). The effects of these basic proteins in the insulin

Fig. 6. Polylysine-dependence of phosphorylation of calmodulin in the presence of insulin

Phosphorylation assays were performed as described in the Experimental section in the presence of insulin and the various concentrations of polylysine indicated. Incorporation of ^{32}P into calmodulin (CaM) was quantified from a representative experiment.

receptor kinase are investigated further in the next paper [22].

The effect of varying the concentration of polylysine on the phosphorylation of calmodulin in the presence of insulin is shown in Fig. 6. A biphasic response occurred,

with initial stimulation of ^{32}P incorporation into calmodulin, followed by inhibition as the polylysine concentration exceeded $0.24\ \mu\text{M}$. In these experiments the concentration of calmodulin is constant. Therefore, the ratio of calmodulin to polylysine is changing, which appears to be important. Maximal ^{32}P incorporation into calmodulin was observed at a molar ratio of calmodulin to polylysine of 5:1. A similar biphasic response was obtained when protamine sulphate was used instead of polylysine, but maximal phosphorylation occurred at a molar ratio of calmodulin to protamine sulphate of approx. 1:15 (results not shown).

Calmodulin-concentration dependence of the phosphorylation of calmodulin

Increasing the calmodulin concentration (while maintaining a constant molar ratio of calmodulin to polylysine of 5:1) resulted in increased ^{32}P incorporation up to $1.8\ \mu\text{M}$ (results not shown). The K_m is approx. $0.1\ \mu\text{M}$ -calmodulin.

Phosphoamino acid analysis and electrophoretic migration of phosphorylated calmodulin

Phosphorylation of calmodulin by purified placental insulin receptors resulted in incorporation of ^{32}P exclusively on to tyrosine residues. The residues which were phosphorylated were independent of the cofactor used, as the results obtained in the presence of protamine sulphate (Figs. 7a and 7c) were identical to those observed

Fig. 7. Phosphoamino acid analysis of calmodulin

Phosphorylation reactions were performed as described in the Experimental section in the presence of $5\ \mu\text{M}$ -protamine sulphate (a and c), or $0.24\ \mu\text{M}$ -polylysine (b and d) and in the presence (a and b) or absence (c and d) of $6.7 \times 10^{-8}\ \text{M}$ -insulin. Acid hydrolysis and phosphoamino acid analysis were performed as described in the Experimental section. The retention times for phosphothreonine (P-Thr), phosphotyrosine (P-Tyr) and phosphoserine (P-Ser) were determined by simultaneous u.v. detection of phosphorylated internal standards. The tracings are representative of two and three separate determinations for protamine sulphate and polylysine respectively.

Fig. 8. Binding of calmodulin to the insulin receptor and polylysine

(a) Calmodulin binds the insulin receptor. Auto-phosphorylation of the insulin receptor and binding to calmodulin–Sepharose were performed as described in the Experimental section. A representative autoradiogram with the starting material (control), the material collected before elution with Ca^{2+} (non-bound), the material eluted with extensive Ca^{2+} washing (Ca^{2+} eluate) and the material eluted with EGTA (EGTA eluate) is shown. The position of migration of the β -subunit of the insulin receptor (β) is indicated. (b) Binding of polylysine to calmodulin. Binding studies were performed as described in the Experimental section. ¹²⁵I-Calmodulin was added to polylysine–Sepharose, allowed to react, then buffer (polylysine), 1 M-NaCl or 0.5 mg of unlabelled calmodulin was added as indicated on the Figure. Results are the means of two separate experimental determinations. The bar labelled ‘Sepharose’ was obtained from incubations performed in the presence of Sepharose not linked to polylysine. Results are expressed as a percentage of the c.p.m. bound to

with polylysine (Figs. 7b and 7d). Similarly, in the absence of insulin (Figs. 7c and 7d), both polylysine and protamine sulphate produced ³²P incorporation exclusively on tyrosine residues. These data indicate that insulin, polylysine and protamine sulphate induce phosphorylation of calmodulin exclusively by stimulating the purified insulin receptor tyrosine kinase.

The characteristic Ca^{2+} -shift in the migration of non-phosphorylated calmodulin on PAGE does not occur with phosphotyrosylcalmodulin [35], consistent with a functional alteration of the phosphorylated species. Identical observations were made in the current study with calmodulin phosphorylated by highly purified placental insulin receptors (results not shown).

Stoichiometry

Utilizing purified receptors and routine assay conditions, we usually observed incorporation of 0.10–0.30 mol of phosphate/mol of calmodulin. This contrasts with the generally lower stoichiometry (0.01–0.10 mol of phosphate/mol of calmodulin) routinely observed using partially purified insulin receptors from adipocytes [35], hepatocytes [20] or skeletal muscle [21]. When assay conditions were optimized by increasing the number of receptors added, increasing the ATP concentration to 500 μM (to avoid substrate depletion) and continuing incubation for 2 h, we obtained incorporation of approx. 1 mol [0.90 ± 0.14 ($n = 5$)] of phosphate/mol of calmodulin. This appears to be the level of saturation, as doubling the number of receptors in the assay did not increase this stoichiometry.

Molecular interactions among polylysine, calmodulin and the insulin receptor

To begin determining the mechanism of action of polylysine, we studied the molecular interactions between calmodulin and the insulin receptor, calmodulin and polylysine and polylysine and the insulin receptor [22]. Previous studies have demonstrated that insulin receptors partially purified from rat adipocytes bind calmodulin [36]. Here we show (Fig. 8a) that the phosphorylated β -subunit of the highly purified placental insulin receptor also binds to calmodulin. Binding of proteins or peptides to calmodulin in the presence of Ca^{2+} and dissociation with EGTA is a standard method for isolating specific calmodulin-binding proteins [37,38]. Lane 1 (Fig. 8a) represents the autophosphorylated β -subunit of the placental insulin receptor that was applied to the calmodulin column in the presence of Ca^{2+} . Although a small amount (9%, as determined by counting the phosphorylated bands) of β -subunit did not bind (lane 2), the majority was specifically bound by calmodulin and was not eluted with Ca^{2+} washes (lanes 3 and 4). When washed with EGTA (lane 5), the phosphorylated β -subunit that was specifically bound to calmodulin in the presence of Ca^{2+} was eluted. This represents 34% of the bound phosphorylated β -subunit. The fraction of the autophosphorylated β -subunit not recovered in the EGTA eluates (66% of the bound subunit) was recovered from the column by washing with SDS. This last fraction probably represents phosphorylated insulin receptor that binds calmodulin with a very high affinity. Although no

polylysine with only buffer added. In these assays, 0.5 mg of calmodulin represents a final concentration of approx. 200 μM .

β -subunit was eluted during Ca^{2+} washing, higher and lower molecular mass phosphoproteins were eluted (lane 3). These were not present in the starting material, and phosphate may have been incorporated when the proteins were bound non-specifically to the Sepharose. These are probably albumin and aggregates of albumin molecules that were used to prepare the column. Further Ca^{2+} washes removed these non-specifically bound proteins (lane 4).

In an attempt to elucidate the molecular interactions between calmodulin and polylysine, we observed that ^{125}I -calmodulin bound to polylysine, and 47% was displaced by the addition of 0.5 mg of unlabelled calmodulin (Fig. 8b). This displacement was concentration-dependent, with 2 mg of unlabelled calmodulin displacing 65% of the bound fraction (results not shown). The bound calmodulin was also displaced by NaCl, with 1 M-NaCl displacing 53% of the bound calmodulin. Addition of 5 mM-EGTA had no effect on calmodulin binding to polylysine, indicating that this is not a Ca^{2+} -dependent process (results not shown). Although proteins are known to bind non-specifically to Sepharose [39], less than 0.1% of the ^{125}I -calmodulin bound to Sepharose that contained no polylysine (Fig. 8b). Similarly, the auto-phosphorylated β -subunit of the insulin receptor exhibits specific binding to polylysine [22].

DISCUSSION

Many of the characteristics of the phosphorylation of calmodulin in the human placental insulin receptor preparation are similar to those observed in rat adipocyte, hepatocyte and skeletal muscle receptor preparations. The near-absolute requirement for Mg^{2+} and the Ca^{2+} -concentration dependence resemble the results obtained with hepatocyte and muscle insulin receptors. Also, calmodulin is phosphorylated predominantly on tyrosine residues in all four systems, indicating that it is phosphorylated directly by the insulin receptor tyrosine kinase. Furthermore, in all systems there is an absolute requirement for certain basic protein cofactors for phosphorylation of calmodulin to occur. Because insulin stimulates the phosphorylation of calmodulin in the intact cell, the molecular interactions among the proteins that are required for the insulin receptor kinase to phosphorylate calmodulin (i.e. calmodulin, polylysine and the insulin receptor) are of considerable interest. The precise role played by the basic proteins has not yet been determined, but we provide evidence here that polylysine binds both to calmodulin (Fig. 8a) and to the insulin

receptor [22], and high salt concentrations partially disrupt the interactions. Furthermore, we confirm previous observations made with partially purified adipocyte receptors [36] that the insulin receptor binds calmodulin in a Ca^{2+} -dependent manner (Fig. 8a). One of the functional consequences of the binding of polylysine to the receptor is insulin-independent activation of the kinase (see [22] for possible binding sites and the effects produced by the interaction of polylysine with the receptor).

The binding of basic proteins to calmodulin has received considerable attention. Previous studies have shown that calmodulin binds a number of basic proteins, including histone H2B [40,41], myelin basic protein [41,42], protamine [42], polyarginine [40] and salmine [40]. Malencik & Anderson [40] reported that polylysine of average molecular mass 4 kDa did not bind to calmodulin while polyarginine of molecular mass 60 kDa did bind to calmodulin. We have demonstrated here that polylysine (of average molecular mass 30 kDa) binds in a non- Ca^{2+} -dependent manner to calmodulin. It has been reported that the minimum structural requirement of a high-affinity ligand for calmodulin is the presence of a basic amphiphilic α -helix [43]. Such highly basic lysine-rich peptides which have basic amphiphilic α -helices bind to calmodulin with high affinity ($K_d \leq 3 \text{ nM}$) [44]. The displacement of calmodulin from polylysine by NaCl may be due to changes in the α -helical and β -sheet helical conformation of the basic peptide induced by the salt [33]. This is supported by the demonstration that increasing the ionic strength (using NaCl) results in a breakdown of the interaction between polylysine and other basic proteins, such as connective tissue glycosaminoglycans [45].

Although the exact binding site for basic peptides is not known, there are a number of highly acidic regions in the calmodulin molecule (Table 1) that may interact with the basic residues of polylysine. Polylysine may bind to one or several of these regions. Analysis of electrostatic potential surfaces suggest that a probable binding site for basic amphiphilic α -helical peptides exists in the second domain of calmodulin (residues 19–32) [46]. This corresponds to the domain designated A-I in Table 1. Another potential binding site for polylysine (A-III) is in the central helix of calmodulin [47]. It has been suggested that this region serves as a flexible tether between the calmodulin lobes [48] and may play an important role in the interaction of calmodulin with receptor proteins and drugs [47]. Binding of polylysine at this site might alter

Table 1. Domains of calmodulin that may interact with polylysine

The calmodulin primary sequence reported by Babu *et al.* [47] is used. Values in parentheses are acidic or basic amino acids as percentages of total amino acids.

Domain	Sequence	Amino acid composition		
		Total	Acidic (E + D)	Basic (R + K)
A-I (1–24)	ADQLTEEQIAEFKEAFSLFDKDG	24	4 + 4 (33.3)	0 + 2 (8.3)
A-II (54–67)	EVDADGNGTIDFPE	14	2 + 3 (35.7)	0 + 0 (–)
A-III (78–87)	DTDSEEEIRE	10	4 + 2 (60)	1 + 0 (10)
A-IV (118–127)	DEEVDEMIRE	10	4 + 2 (60)	1 + 0 (10)

the relative position of the two lobes [48]. With respect to the important role of polylysine-like molecules as cofactors for the receptor kinase, we have recently demonstrated that the highly basic C-terminal fragment of the human c-Ki-ras 2 gene product (Lys-Lys-Lys-Lys-Lys-Lys-Ser-Lys-Thr-Lys-Cys-Val-Ile-Met), a membrane-bound protein, obviates the need for other basic peptides [49]. Intact purified v-Ki-ras protein, which contains a highly basic domain in the C-terminal region, is phosphorylated by purified insulin receptor kinase without exogenous basic cofactors. In contrast, purified H-ras proteins (wild and mutant types) which do not have an integral highly basic region, are phosphorylated by insulin receptor kinase only in the presence of polylysine [50].

The tyrosine kinase in the purified receptor preparation was much more effective than the kinase in the WGA-Sephadex preparations in stimulating the incorporation of ^{32}P into calmodulin. Under optimum conditions, we were able to obtain maximal incorporation of 1.0 mol of phosphate/mol of calmodulin with the purified receptor preparation, but were unable to achieve this with partially purified receptors. This maximum stoichiometry is consistent with our previous observation that calmodulin is phosphorylated by the insulin receptor kinase exclusively on tyrosine residue 99, located in the third Ca^{2+} -binding domain of the molecule [35].

In summary, we have demonstrated that calmodulin is a substrate for the purified insulin receptor tyrosine kinase of human placenta. The low K_m for calmodulin compares favourably with that observed for other substrates of the receptor tyrosine kinase [51]. Although the reaction is similar in many respects to that observed with partially purified rat adipocyte, hepatocyte and skeletal muscle insulin receptors, the highly purified placental receptor is far more effective at phosphorylating calmodulin. Basic proteins alone, such as polylysine, are more potent activators of the kinase in the highly purified receptors than in partially purified receptors, and are shown to bind to calmodulin by electrostatic interactions. Phosphotyrosylcalmodulin behaves differently from non-phosphorylated calmodulin in that it fails to exhibit the characteristic Ca^{2+} -shift on electrophoresis and exhibits altered elution characteristics in response to Ca^{2+} on anion-exchange h.p.l.c. [52]. These observations indicate that the interaction between Ca^{2+} and phosphotyrosylcalmodulin is altered. Further characterization of this post-translational modification of calmodulin and of the molecular mechanisms responsible for kinase activation and substrate coupling are necessary to determine whether it plays a specific role in the mechanism of insulin action.

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