Effect of basic polycations and proteins on purified insulin receptor

Insulin-independent activation of the receptor tyrosine-specific protein kinase by poly(L-lysine)

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Since the studies on tyrosine phosphorylation of calmodulin by the insulin receptor kinase *in vitro* suggested that protamine and poly(L-lysine) may activate phosphorylation of the receptor β subunit [Sacks & McDonald (1988) *J. Biol. Chem.* **263**, 2377–2383], we examined the effects of a variety of basic polycations/proteins and polyamines on insulin receptor kinase activity. The insulin receptor purified from human placental membranes was incubated with each basic polycation/protein or polyamine and assayed for tyrosine-specific protein kinase activity by measuring ^{32}P incorporation into the *src*-related peptide. At a concentration of $1\ \mu\text{M}$, poly(L-lysine) and poly(L-ornithine) markedly stimulated kinase activity, whereas poly(L-arginine) and histones H1 and H2B inhibited insulin receptor kinase. In contrast, at a concentration of $1\ \text{mM}$, three polyamines (spermine, spermidine and putrescine) did not alter kinase activity. Poly(L-lysine) and poly(L-ornithine) stimulated the insulin receptor kinase by 5–10-fold at concentrations of 0.1 – $1\ \mu\text{M}$. Protamine sulphate also showed a significant stimulatory effect at a concentration of $100\ \mu\text{M}$. Preincubation of the receptor with poly(L-lysine) or poly(L-ornithine) for 20–60 min resulted in maximal kinase activation. Poly(L-lysine), the most effective activator of the receptor kinase, was used to characterize further the mechanisms of the kinase activation. Poly(L-lysine) activates the insulin receptor kinase by increasing the V_{max} without changing the K_m . Poly(L-lysine) markedly stimulates the kinase activity of insulin receptor preparations that have lost both basal kinase activity and the ability to be stimulated by insulin. Insulin and poly(L-lysine) also differed in their ability to stimulate the kinase activity of prephosphorylated receptors. Prephosphorylation of the receptors did not affect the stimulation of the kinase by insulin. In contrast, prephosphorylation of receptors resulted in a markedly enhanced ability of poly(L-lysine) to stimulate kinase activity. These studies suggest that the mechanisms by which poly(L-lysine) and insulin activate the kinase are different. In conjunction with other additional evidence, it is suggested that poly(L-lysine) interacts directly with the β -subunit of the receptor, thereby activating the receptor kinase.

INTRODUCTION

The insulin receptor is a tyrosine-specific protein kinase [1–3] which is activated when insulin binds to the extracellular domain of the receptor. The tyrosine protein kinase activity of the insulin receptor is thought to mediate some or all of the actions of insulin [3]. Since insulin promotes phosphorylation and dephosphorylation of proteins in its target cells [4], it is tempting to speculate that the insulin receptor tyrosine kinase initiates the signal cascade. To date, however, the signal pathways leading to intracellular sites of insulin action have not been identified.

Inhibitors or activators of tyrosine-specific protein kinases can be useful tools, not only in the biochemical characterization of these recently discovered enzymes, but also for understanding their physiological roles *in vivo*. Inhibitors thus far reported include low molecular mass compounds such as amiloride [5], genistein [6] and staurosporine [7], and some types of tyrosine-containing

synthetic polymers [8]. The insulin receptor kinase can be activated by several mechanisms. (i) Insulin, by binding to the outer surface domain, activates the cytoplasmic kinase, which in turn phosphorylates itself. This autophosphorylation process further activates the receptor kinase [9]. (ii) Mild dithiothreitol treatment of the receptor activates kinase activity [10,11]. (iii) Mild trypsin treatment activates the insulin receptor kinase [12,13]. (iv) The kinase of a membrane-anchored insulin receptor kinase domain expressed in Chinese hamster ovary cells is constitutively activated [14]. It is likely that the kinase activity of the intact $\alpha_2\beta_2$ receptor is suppressed and that insulin, dithiothreitol and trypsin activate the kinase by changing the conformation of the intact receptor to kinase-active form(s) without direct interaction with the cytoplasmic tyrosine kinase domains.

The insulin receptor kinase is labile, readily losing activity during purification and storage. Our previous studies suggested that the C-terminus of the β -subunit may be involved in the regulation of the tyrosine kinase

Abbreviation used: PAGE, polyacrylamide-gel electrophoresis.

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activity [15]. Whereas the intact 90 kDa β -subunit has full kinase activity, degraded forms such as the 88 kDa β -subunit show little kinase activity. Inactivation of the kinase appears to arise from a conformational change in the 90 kDa form, which with time goes into a 'relaxed conformation', becoming insensitive to insulin. This change appears to make the receptor susceptible to proteolysis at the C-terminal end of the β -subunit. The process is accelerated when the receptor is stored at 4 or 37 °C. Although freezing the receptor at -70 °C in the presence of 10% glycerol significantly improves the stability of the kinase, a decrease in activity accompanied by loss of stimulation by insulin is still routinely observed.

Calmodulin is phosphorylated in intact rat adipocytes in an insulin-dependent manner [16]. Phosphorylation of calmodulin by insulin receptor kinase purified from human placentas [17] or partially purified from liver [18] or skeletal muscle [19] requires the presence of cofactors such as poly(L-lysine) or protamine sulphate. During the course of these studies, we observed that these basic protein cofactors stimulated the autophosphorylation of the β -subunit of the receptor independently of insulin [17,18]. There, we have further characterized the direct effects of these and other selected basic polycations/proteins on receptor autophosphorylation and kinase activity as measured by incorporation of ^{32}P into the *src*-related peptide substrate. We observed that some of the basic polycations/proteins themselves activate insulin receptor kinase. This activation is much greater than that by insulin and appears to be independent of insulin. In addition, poly(L-lysine) can stimulate the kinase activity of receptor preparations which do not exhibit insulin-sensitive kinase activity. These observations, in conjunction with other additional evidence, suggest that poly(L-lysine) interacts directly with the receptor β -subunit domain, thereby activating the receptor kinase.

Part of this work was presented at the FASEB meeting in May 1988 [19a].

EXPERIMENTAL

Materials

Poly(L-lysine) (30 000–70 000 kDa; mean 41 000 kDa), poly(L-ornithine) (15 000–30 000 kDa; mean 25 000 kDa), poly(L-arginine) (15 000–70 000 kDa; mean 41 000 kDa), protamine sulphate, putrescine, spermine, spermidine, histone H1, phosphoamino acids and Sepharose CL-6B were obtained from Sigma. Histone H2B was from Worthington Biochemical Co. Crystalline pig insulin was kindly supplied by Eli Lilly. Molecular mass markers and reagents for SDS/polyacrylamide-gel electrophoresis (SDS/PAGE) were obtained from Bio-Rad. A synthetic peptide resembling the tyrosine phosphorylation site of pp60^{src} (Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly) was purchased from Peninsula Laboratories [20]. [γ - ^{32}P]ATP was from New England Nuclear.

Purification of the receptor

Insulin receptor was purified from Triton X-100 solubilized human placental membranes by sequential affinity chromatography on wheat-germ agglutinin (WGA)- and insulin-Sepharose as previously described [21].

Phosphorylation of an exogenous substrate (kinase assay)

Phosphorylation assays were carried out at 25 °C for 40 min in 30 μl of 50 mM-Tris/HCl buffer, pH 7.4, containing 1 mM of the *src*-related peptide, 2 mM-MnCl₂, 15 mM-MgCl₂, 0.1% (v/v) Triton X-100 and 40 μM -[γ - ^{32}P]ATP (~ 12000 c.p.m./pmol) and the purified insulin receptor (~ 0.1 μg), which had been preincubated with insulin or one of the basic polycations/proteins at 25 °C for 1 h. The reaction was terminated by the addition of 50 μl of 5% (w/v) trichloroacetic acid and 20 μl of bovine serum albumin (1 mg/ml). After incubating this solution at 0 °C for 30 min, the proteins were precipitated by centrifugation. Duplicate 35 μl aliquots of the supernatant were spotted on to pieces of phosphocellulose paper (Whatman, P-82). The papers were extensively washed in 75 mM-phosphoric acid. Incorporation of ^{32}P into the peptide was quantified by counting in a liquid scintillation counter.

Phosphorylation of the β -subunit

Phosphorylation of the purified receptor was carried out in the same manner as described for the exogenous substrate phosphorylation. The reaction was stopped by the addition of 3 $\times$ concentrated Laemmli [22] sample buffer, followed by boiling for 5 min. SDS/PAGE was performed according to Laemmli [22] under reducing conditions. The gels were stained with silver, dried and autoradiographed.

Phosphoamino acid analysis

The radioactive bands corresponding to phosphorylated β - and α -subunits were excised, washed, eluted by trypsin digestion as described previously [23] and hydrolysed in 6 M-HCl for 2 h at 100 °C. The hydrolysates were analysed by paper electrophoresis (pH 3.5, 2000 V, 1 h) and autoradiography.

Binding of placental insulin receptor to polylysine-Sepharose

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 buffer, pH 7.5, then incubated with 5 ml of autophosphorylated purified insulin receptor for 10 min at 22 °C. Additions were made as indicated in the Fig. 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 buffer, pH 7.5, the radioactivity bound to the polylysine-Sepharose was determined. Sepharose which had no polylysine attached was used as a control.

RESULTS

Effects of various basic polycations/proteins on insulin receptor kinase activity

Tyrosine-specific protein kinase activity of the purified insulin receptor was assayed by measuring incorporation of ^{32}P into the *src*-related peptide after the receptor was preincubated, in the absence of insulin, with 1 μM of a variety of basic polycations/proteins or polyamines, i.e. poly(L-lysine), poly(L-arginine), poly(L-ornithine), protamine sulphate, histone H1, histone H2B, putrescine, spermine or spermidine. The results are summarized in Fig. 1, in which the rate of kinase activity is expressed as a percentage of the activity achieved in the absence of

Fig. 1. Effects of basic polycations/proteins or polyamines on insulin receptor kinase activity

Purified insulin receptor (approx $0.1 \mu\text{g}$) was preincubated with $1 \mu\text{M}$ of the basic proteins or polyamines, as indicated, at 25°C for 1 h in $20 \mu\text{l}$ of 50 mM -Tris/HCl buffer, pH 7.4, containing 0.1% (v/v) Triton X-100. Tyrosine kinase activity of the receptor was then assayed by measuring incorporation of ^{32}P into the *src*-related peptide as described in the Experimental section. The rate of kinase activity of each sample (pmol/min) is expressed as a percentage of the activity achieved in the presence of $1 \mu\text{M}$ -poly(L-lysine). Results shown are the means and S.D. of three independent experiments using the same insulin receptor preparation. Control represents the basal kinase activity which is the activity of the receptor preincubated in the Tris/HCl buffer containing neither basic proteins nor insulin.

poly(L-lysine). At this concentration, poly(L-lysine) and poly(L-ornithine) activated the kinase by approx 5- and 4-fold over basal activity (control) respectively, whereas the other compounds showed either inhibition or no effect. The three polyamines did not have any effect on kinase activity even at higher concentrations (1 mM). Insulin binding activity was not affected by $1 \mu\text{M}$ -poly(L-lysine) or $1 \mu\text{M}$ -poly(L-ornithine) (results not shown).

Concentration-dependence for activation of insulin receptor kinase by basic polycations/proteins

Dose-response curves for four proteins which either stimulated [poly(L-lysine) and poly(L-ornithine)] or inhibited [poly(L-arginine) and protamine sulphate] the kinase activity at a concentration of $1 \mu\text{M}$ were examined next (Fig. 2). Poly(L-lysine) stimulated kinase activity at concentrations of 0.1 – $1 \mu\text{M}$, but had no effect at $10 \mu\text{M}$. Poly(L-ornithine) was stimulatory at 0.1 – $10 \mu\text{M}$, but exhibited no effect at $100 \mu\text{M}$. Protamine sulphate showed stimulation only at higher concentrations, approx. 2-fold at $10 \mu\text{M}$ and 3.5-fold at $100 \mu\text{M}$. In contrast, poly(L-arginine) inhibited the kinase activity at all concentrations examined. Although not studied, it is possible that poly(L-lysine) or poly(L-ornithine) may also inhibit kinase activity at higher concentrations.

Fig. 2. Effects of varying concentrations of basic polycations/proteins on insulin receptor kinase activity

Dose-response curves for four proteins which either stimulated or inhibited kinase activity (Fig. 1) are presented. Purified insulin receptor (approx $0.1 \mu\text{g}$) was preincubated with different concentrations of poly(L-lysine) ($\circ$), poly(L-ornithine) ($\bullet$), protamine sulphate ($\triangle$) or poly(L-arginine) ($\blacktriangle$). Tyrosine kinase activity of the receptor was then assayed by measuring ^{32}P incorporation into the *src*-related peptide as described in the Experimental section. The rate of kinase activity is expressed as a percentage of basal kinase activity, which is the activity of purified insulin receptor preincubated in the Tris/HCl buffer containing neither basic polycations/proteins nor insulin. Each point represents either the mean and S.D. of four independent experiments, or the mean value of two experiments.

Time-dependence for activation of insulin receptor kinase by poly(L-lysine) or poly(L-ornithine)

Insulin receptor kinase activity was assayed after preincubation of the receptor with either $0.24 \mu\text{M}$ -poly(L-lysine) or $1 \mu\text{M}$ -poly(L-ornithine) for the time intervals indicated in Fig. 3. Preincubation for 20–60 min was necessary to achieve maximal kinase activation by either poly(L-lysine) or poly(L-ornithine). Prolonged incubation appeared to result in a reduced kinase activation.

Comparison of the effects of poly(L-lysine) and insulin on insulin receptor kinase activity and autophosphorylation of the β -subunit

In order to compare activation of the kinase by poly(L-lysine) with that by insulin, the effects of different concentrations of poly(L-lysine) on kinase activity were examined in the absence or presence of $1 \mu\text{M}$ -insulin. As shown in Fig. 4, the stimulatory effects of insulin and poly(L-lysine) were additive and independent of each other. Note that insulin activated the kinase to the same extent in the control [no poly(L-lysine)] as in the presence of $100 \mu\text{M}$ -poly(L-lysine), where the stimulatory effect of poly(L-lysine) was no longer observed. Moreover, the maximum kinase activation by poly(L-lysine) was much greater than that by insulin.

In parallel studies to those described above, the effects of insulin and poly(L-lysine) on receptor autophosphorylation were examined. As seen in Fig. 5(a), both insulin and poly(L-lysine) stimulated phosphorylation of the β -subunit (lanes 2 and 3). The effects of different concentrations of poly(L-lysine) on phosphorylation of the β -

Fig. 3. Effects of varying preincubation intervals with poly(L-lysine) or poly(L-ornithine) on insulin receptor kinase activity

Purified insulin receptor (approx. 0.1 μ g) was preincubated with either 0.24 μ M-poly(L-lysine) (○) or 1 μ M-poly(L-ornithine) (●) for the indicated periods. Tyrosine kinase activity of the receptor was then assayed by measuring 32 P incorporation into the *src*-related peptide as described in the Experimental section. The rate of kinase activity is expressed as a percentage of the basal kinase activity. Each point represents the mean value of duplicate experiments.

Fig. 4. Effects of varying concentrations of poly(L-lysine) on insulin receptor kinase activity in the absence and presence of insulin

Purified insulin receptor (approx. 0.1 μ g) was preincubated at 25 $^{\circ}$ C for 1 h with various concentrations of poly(L-lysine) in the absence (open bars) or presence (closed bars) of 1 μ M-insulin in 20 μ l of 50 mM-Tris/HCl buffer, pH 7.4, containing 0.1% (v/v) Triton X-100. Tyrosine kinase activity of the receptor was then assayed by measuring 32 P incorporation into the *src*-related peptide as described in the Experimental section. The rate of kinase activity is expressed as a percentage of the basal kinase activity, which is the activity of purified insulin receptor

Fig. 5. Effects of poly(L-lysine) or insulin on phosphorylation of the insulin receptor subunits

(a) SDS/PAGE analysis of the phosphorylated receptor. Purified insulin receptor (approx. 1 μ g) was preincubated with Tris/HCl buffer (lane 1), 1 μ M-insulin (lane 2) or 0.24 μ M-poly(L-lysine) (lane 3). Phosphorylation of the receptor was then carried out as described in the Experimental section. After the phosphorylation reaction, the receptor was analysed by SDS/PAGE (10% gel) under reducing conditions. The gel was exposed to a film (Kodak XAR-5) for 45 min at -70° C with an intensifying screen. (b) Phosphoamino acid analysis of the β - and α -subunits of the insulin receptor phosphorylated in the presence or absence of insulin or poly(L-lysine). The bands corresponding to phosphorylated β - and α -subunits shown in (a) were excised, washed, eluted by trypsin digestion as described previously [23] and hydrolysed in 6 M-HCl for 2 h at 110 $^{\circ}$ C. The hydrolysates were analysed by paper electrophoresis (pH 3.5, 2000 V, 1 h) and autoradiography. Lane 1 (760 c.p.m.), β -subunit basal phosphorylation; lane 2 (1600 c.p.m.), insulin-stimulated β -subunit phosphorylation; lanes 3 (4400 c.p.m.) and 4 (1000 c.p.m.), poly(L-lysine)-stimulated phosphorylation of β - and α -subunit respectively. The radioactive samples were electrophoresed together with three phosphoamino acids and stained with ninhydrin (corresponding ninhydrin spots are indicated by the arrows). X indicates the origin.

subunit in the absence or presence of 1 μ M-insulin were consistent with those on kinase activity (results not shown). In contrast to insulin, poly(L-lysine) stimulated phosphorylation of the α - and β_1 -subunits in addition to the β -subunit. (The β_1 -subunit, which is often seen in purified insulin receptor preparations [21], is the *N*-terminal portion of the β -subunit generated by proteolytic loss of the kinase domain [2,23].

Phosphoamino acid analysis was performed on phosphorylated subunits of the insulin receptor. (Note that the kinase activity described in this paper reflects

preincubated in the Tris/HCl buffer containing neither poly(L-lysine) nor insulin. Shown are the means of two independent experiments using the same receptor preparation.

tyrosine-specific kinase activity, since the substrate, *src*-related peptide, contains only tyrosine as a potential phosphorylation site.) Both insulin (Fig. 5b, lane 2) and poly(L-lysine) (Fig. 5b, lanes 3 and 4) stimulated phosphorylation of insulin receptor subunits on tyrosine residues. The α -subunit, phosphorylated in the presence of poly(L-lysine) but not insulin, had phosphate incorporated exclusively on tyrosine residues (lane 4). In contrast, both poly(L-lysine) (lane 3) and insulin (lane 2) induced a small amount of serine phosphorylation in the β -subunit. This suggests that there is a serine kinase in this purified receptor preparation. Previous studies with similar receptor preparations [24,25], however, did not reveal ^{32}P incorporation into serine residues.

Activation of partially inactivated insulin receptor kinase by poly(L-lysine)

Our collective observations made over the last five years with a number of purified insulin receptor preparations indicate that the kinase activity is unstable. During storage at -70°C , basal kinase activity initially increases slightly, later becoming inactivated with a concomitant and progressive loss of insulin sensitivity. We hypothesized that poly(L-lysine) might be able to activate insulin receptor kinase which has reduced basal activity and is insulin-insensitive. To test this possibility, purified insulin receptor preparations stored at -70°C for various periods of time were examined.

Table 1 shows representative examples of kinase activity of different receptor preparations (stored up to 36 months) after preincubation with $1\ \mu\text{M}$ -insulin, $0.24\ \mu\text{M}$ -poly(L-lysine) or buffer (basal). Poly(L-lysine) activated insulin receptor preparations which had lost both basal and insulin-stimulative kinase activities (preparation A,

Fig. 6. Effects of insulin or poly(L-lysine) on insulin receptor autophosphorylation after storage at -70°C

Freshly prepared insulin receptor (3 days old, lanes 10–12) or purified receptor preparations (IR prep.) stored at -70°C for indicated period in months (lanes 1–12) were incubated with $1\ \mu\text{M}$ -insulin (lanes 1, 4, 7 and 10), no additions (lanes 2, 5, 8 and 11) or $0.24\ \mu\text{M}$ -poly(L-lysine) (lanes 3, 6, 9 and 12). Phosphorylation was then carried out as described in the Experimental section. The receptor was analysed by SDS/PAGE (7.5% gel) under reducing conditions. The gel was stained with silver, dried and autoradiographed.

Table 1. Effects of poly(L-lysine) and insulin on kinase activity of highly purified insulin receptor preparations after storage at -70°C

Freshly prepared insulin receptor (E, less than 3 days' storage) as well as receptor preparations stored at -70°C for different lengths of time (A–D) were preincubated with $1\ \mu\text{M}$ -insulin, $0.24\ \mu\text{M}$ -poly(L-lysine), or no additions (basal) and then assayed for kinase activity as described in the Experimental section.

Insulin receptor preparation	Period of storage at -70°C	Preincubated with:	Kinase activity (pmol/min)
A	36 months	Insulin	0
		Basal	0
		Poly(L-Lys)	0.001
B	28 months	Insulin	0.009
		Basal	0.009
		Poly(L-Lys)	0.058
C	15 months	Insulin	0.014
		Basal	0.005
		Poly(L-Lys)	0.076
D	3 months	Insulin	0.061
		Basal	0.018
		Poly(L-Lys)	0.384
E	'Fresh' (< 3 days)	Insulin	0.052
		Basal	0.006
		Poly(L-Lys)	0.377

36 months old) or had lost only insulin-stimulative kinase activity (preparation B, 28 months old) during storage. Preparations older than 36 months did not show any kinase activity even after treatment with poly(L-lysine) (results not shown), suggesting that poly(L-lysine) can activate insulin receptor kinase which is partially, but not completely, inactivated. The degree of kinase activation by poly(L-lysine) ranged from 6- to 60-fold.

The effects of poly(L-lysine) or insulin on phosphorylation of insulin receptor subunits are shown in Fig. 6. Insulin receptor preparations in lanes 1–3, 4–6, 7–9 and 10–12 were the same as those designated A, B, C and E in Table 1. The results of autophosphorylation in the presence of insulin or poly(L-lysine) correlate well with those of kinase activity summarized in Table 1.

Potential mechanisms of insulin receptor kinase activation by poly(L-lysine)

Kinetic analysis revealed that poly(L-lysine) activates the insulin receptor kinase by increasing the V_{max} , without changing the K_m towards the peptide (Fig. 7). This activation could be caused by an interaction between poly(L-lysine) and the *src*-related peptide substrate. This is unlikely, since the K_m for the *src*-related peptides does not change significantly in the presence of poly(L-lysine). Both peptides are basic and would probably not interact. C.d. analysis (200–320 nm) suggests that the *src* peptide (0.1 mM) does not have an ordered secondary structure in 50 mM-Tris/HCl buffer, pH 7.4, and the addition of

Fig. 7. Kinetic analysis of the insulin receptor kinase

Purified receptor (approx. 0.1 μ g) was preincubated with 1 μ M-insulin ($\bullet$), 0.24 μ M-poly(L-lysine) ($\times$) or no additions ($\circ$) at 25 $^{\circ}$ C for 1 h. Kinase activity was then measured at various concentrations of the *src*-related peptide. The results of a fresh receptor preparation (a and b) are presented. Panel (b) is a double reciprocal plot of the data shown in (a).

0.1 μ M-poly(L-lysine) does not change its c.d. spectrum (results not shown). (Although not studied, it is possible that the inhibitory effects of basic proteins are due to non-specific interactions with the *src*-related peptide.)

In an attempt to elucidate the molecular interactions between the insulin receptor and poly(L-lysine), the autophosphorylated β -subunit was applied on to polylysine-Sepharose in the presence of buffer, 1 M-NaCl or 0.5 mg of calmodulin (Fig. 8). The β -subunit bound to the polylysine-Sepharose. Addition of 1 M-NaCl displaced 60% of the bound β -subunit, while 0.5 mg ($\sim 20 \mu$ M) of calmodulin displaced 95%. The same concentration of bovine serum albumin (200 μ M) had no effect on binding (results not shown). The specificity of binding to poly(L-lysine) was confirmed by the observation that less than 0.1% of the autophosphorylated β -subunit bound to Sepharose alone.

The aforementioned evidence suggests that activation of the kinase by poly(L-lysine) occurs via a direct interaction between the polycation and the insulin receptor. Poly(L-lysine) did not significantly alter the K_m for ATP (results not shown).

Fig. 8. Binding of poly(L-lysine) to the insulin receptor

Binding studies were performed as described in the Experimental section. Autophosphorylated purified placental insulin receptor was added to polylysine-Sepharose, in the presence of buffer ('polylysine'), 1 M-NaCl or 0.5 mg of calmodulin. A representative experiment is shown. 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 polylysine with only buffer added.

Effects of prephosphorylation of the β -subunit on kinase activation and autophosphorylation of the insulin receptor

In order to compare the mechanisms of kinase activation by poly(L-lysine) and insulin, the effect of prephosphorylation of the β -subunit on kinase activation by poly(L-lysine) or insulin was examined (Table 2). Control insulin receptor kinase (not prephosphorylated) was activated 3- and 9-fold by insulin and poly(L-lysine) respectively. Basal activity of prephosphorylated receptor kinase was three times greater than that of control receptor kinase. Incubation with insulin and poly(L-lysine) further activated the prephosphorylated receptor kinase 3- and 31-fold respectively. These results suggest that, while kinase activation by insulin is not affected by prephosphorylation of the β -subunit, poly(L-lysine) activates prephosphorylated receptor kinase more effectively (31-fold) than it activates control receptor kinase (9-fold).

The effect of poly(L-lysine) or insulin on autophosphorylation of the prephosphorylated β -subunit was examined by analysing the receptor preparations derived from the experiments shown in Table 2. Fig. 9 shows autophosphorylation of prephosphorylated receptors (lanes 3–5) and control receptors (lanes 1, 2 and 6) in the presence of insulin (lanes 2 and 4), poly(L-lysine) (lanes 5 and 6) or no additions (basal) (lanes 1 and 3). The β -subunit of prephosphorylated receptors was phosphorylated much more than the control receptors in all

Table 2. Effects of prephosphorylation of the insulin receptor on kinase activity

For prephosphorylated receptor experiments, prephosphorylation was started by the addition of 40 μM - $[\gamma\text{-}^{32}\text{P}]\text{ATP}/15\text{ mM-MgCl}_2/2\text{ mM-MnCl}_2$, and the reaction was allowed to proceed for 60 min at 25 °C. The prephosphorylated receptor was then incubated with 50 mM-Tris/HCl buffer (basal), insulin or poly(L-lysine) for 60 min at 25 °C. The kinase assay was started by the addition of the *src*-related peptide. After 40 min, ^{32}P incorporation into the peptide was measured as described in the Experimental section. For the control experiments, the receptor was incubated with the Tris/HCl buffer for 60 min at 25 °C, and then with the buffer, insulin or poly(L-lysine) for 60 min at 25 °C. Kinase activity was assayed as described in the Experimental section.

Incubation conditions	Insulin receptor kinase activity				
	Non-phosphorylated		Prephosphorylated		
	(pmol/min)	(%)*	(pmol/min)	(%)*	(-fold)†
Basal	0.0102	100	0.0300	294	1
Insulin (1 μM)	0.0317	310	0.0788	773	2.6
Poly(L-lysine) (0.24 μM)	0.0926	908	0.921	9030	30.7

* Activity is expressed as a percentage of the basal activity of non-prephosphorylated receptor.
† Activity is expressed as a -fold increase compared with the basal activity of prephosphorylated receptor.

three cases [basal, insulin and poly(L-lysine)]. The most striking difference was the dramatic increase produced by poly(L-lysine) on activation of the prephosphorylated receptor (Fig. 9, lane 5). As previously noted (Fig. 4), the α -subunit was also phosphorylated in the presence of poly(L-lysine) (lane 5).

DISCUSSION

We have shown that phosphorylation of calmodulin *in vitro* by the insulin receptor kinase from a variety of tissues [17–19, 26] requires the presence of cofactors such as poly(L-lysine) or protamine sulphate. Since insulin-induced phosphorylation of calmodulin is observed in intact rat adipocytes [16], the absolute requirement for cofactors to obtain phosphorylation of calmodulin *in vitro* raises the possibility that some naturally existing factors, which may link calmodulin and insulin receptor kinase, are missing from broken cell systems. Identifying such factors in insulin-sensitive cells will be necessary to reconstitute a biologically relevant insulin receptor-calmodulin system *in vitro*. In this regard, it is important to characterize the role of the basic polycations/proteins in these systems.

There are marked differences between the phosphorylation of calmodulin and other substrates by the insulin kinase. Without any cofactors, the purified insulin receptor kinase is able to phosphorylate some of the purified proteins such as tubulin, microtubule-associated proteins (MAP2 and tau), fodrin and IgG fractions from

Fig. 9. Effects of prephosphorylation of the insulin receptor on insulin- or poly(L-lysine) stimulated phosphorylation of the β -subunit

Purified insulin receptor was incubated initially with 50 mM-Tris/HCl buffer, pH 7.4 (lanes 1, 2 and 6) or 40 μM - $[\gamma\text{-}^{32}\text{P}]\text{ATP}/15\text{ mM-MgCl}_2/2\text{ mM-MnCl}_2$ (prephosphorylated IR) (lanes 3, 4 and 5) for 60 min at 25 °C, and then for 60 min with no additions (lanes 1 and 3; basal), insulin (Ins) (lanes 2 and 4), or poly(L-lysine) (lanes 5 and 6). Kinase assays were initiated by adding the *src*-related peptide and the $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ /metal mixture to control receptors (lanes 1, 2 and 6) and the *src*-related peptide alone to prephosphorylated receptors (lanes 3, 4 and 5). After 40 min at 25 °C, the reaction was terminated as described in the Experimental section. Trichloroacetic acid precipitates of the reaction mixtures (shown in Table 2) were analysed by SDS/PAGE and autoradiography.

Rous sarcoma virus-infected rabbits [25,27,28]. It is reasonable to postulate, therefore, that the basic polycations/proteins interact predominantly with calmodulin, enabling potential tyrosine phosphorylation sites to become accessible to the insulin receptor kinase. On the other hand, these basic polycations/proteins, which were previously shown to stimulate phosphorylation of the β -subunit [18], may influence the kinase activity itself. The present studies were designed to characterize further the direct effects of basic polycations/proteins on the purified insulin receptor tyrosine kinase using a peptide substrate containing only tyrosine as a phosphorylation site.

Some basic polycations/proteins, such as poly(L-lysine), poly(L-ornithine) and protamine sulphate, stimulate autophosphorylation of the receptor and activate the insulin receptor kinase. The concentration-dependence for kinase activation by each basic polycation/protein is different. Kinase activation by poly(L-lysine) is clearly biphasic, with maximum effects at concentrations of 0.1 and 1 μM (Figs. 2 and 4a). Similarly, poly(L-ornithine) produced a biphasic effect on kinase activity, but was effective over a greater range of concentrations, losing its effect at 100 μM (Fig. 2). In contrast, kinase activation by protamine sulphate requires much higher concentrations (> 10 μM). Maximum activation of insulin receptor kinase activity for each of these basic polycations/proteins is obtained at molar ratios of insulin receptors to basic polycations/proteins of approx. 10–100, 10–1000 and > 1000 for poly(L-lysine), poly(L-ornithine) and

protamine sulphate respectively. Interestingly, similar biphasic effects were produced by these basic polycations/proteins when the phosphorylation of calmodulin was studied [17]. The reasons for these biphasic effects are not clear. The decrease in activity at higher concentrations of polycations is not due to limited substrate binding to phosphocellulose papers, since β -subunit phosphorylation activation by poly(L-lysine) was also biphasic.

There are some similarities in the ability of basic polycations/proteins to activate the kinase and act as cofactors for calmodulin phosphorylation: (i) poly(L-lysine), poly(L-ornithine) and protamine sulphate are effective; (ii) the activation by poly(L-lysine) is dose-dependent and biphasic for both processes; and (iii) polyamines such as putrescine, spermine and spermidine show no effects on either process. There are also some differences: (i) histones H1 and H2B, which are good cofactors for calmodulin phosphorylation, inhibit the insulin receptor kinase activity; (ii) protamine sulphate is not as good an activator for the kinase as it is a cofactor for calmodulin phosphorylation; and (iii) poly(L-arginine) is effective as a cofactor for phosphorylation of calmodulin, but does not appear to activate the kinase. Some of these differences may be attributable to differences in the assay conditions. For example, in the receptor kinase assays both Mn^{2+} and Mg^{2+} are present, while the calmodulin phosphorylation assays contain, by necessity, only Mg^{2+} . In addition, in the latter assay Ca^{2+} is maintained in the submicromolar range by Ca^{2+} /EGTA buffers. These buffers are not present in the kinase assays and thus free Ca^{2+} is supramicromolar due to the usual Ca^{2+} contamination of the reagents.

Since poly(L-lysine) was found to be the most effective activator of the purified insulin receptor kinase, it was used as a model to study further the mechanism of kinase activation by basic polycations/proteins. Poly(L-lysine) appears to activate the insulin receptor kinase by a mechanism which is independent of insulin. Poly(L-lysine) is able to markedly activate the kinase activity of insulin receptor preparations that have lost some, but not all, of their basal activity, and some or all of their insulin-sensitivity. In other words, poly(L-lysine) is capable of activating the kinase which has been partially inactivated or is minimally active. It is plausible that poly(L-lysine) places the insulin receptor kinase in a conformation which yields higher specific activity, characterized by an increase in the V_{max} .

Several lines of evidence indicate that poly(L-lysine) interacts with the insulin receptor, which leads to kinase

activation. First, the insulin receptor binds to poly(L-lysine)-Sephadex. Secondly, poly(L-lysine) increases the kinase activity of prephosphorylated receptors (probably by enhancing salt bridges) more than the activity of control receptors. A direct interaction of basic polycations/proteins with the *src*-related peptide can be excluded for the reasons described in the Results section. Acidic domains in the insulin receptor which may potentially interact with poly(L-lysine) are summarized in Table 3. These are A-I (633–645), A-II (695–707) and A-III (1268–1286). Two of these acidic domains, A-I and A-II, are found within 90 amino acid residues of the α -subunit C-terminus. A-III is the most acidic domain we identified in the insulin receptor. It is adjacent to the kinase domain and is located within 80 amino acid residues of the C-terminal domain of the β -subunit. Previously, we have studied the mechanisms responsible for inactivation of the insulin receptor kinase, and postulated that the C-terminus of the β -subunit is an important determinant of the kinase activity of the insulin receptor [15]. Extrapolating from the earlier studies, it is possible that poly(L-lysine) activates the kinase by stabilizing this important C-terminal domain. Of the three domains, therefore, the A-III domain would be the most likely to interact with poly(L-lysine), causing the kinase activation. However, we cannot exclude the possibility that poly(L-lysine) activates the kinase by binding to the domains distant from the kinase domain. As the biphasic effect on kinase activation by poly(L-lysine) indicated, receptor aggregation may also be involved. It is certainly possible that poly(L-lysine), by binding to the acidic domains, potentiates aggregation of the insulin receptor in a manner similar to the effect observed with anti-receptor antibodies [29]. Our radiation inactivation studies on insulin receptor kinase activity suggested that the receptor aggregate, $(\alpha_2\beta_2)_{8-10}$, is a highly active kinase [30]. In view of this, poly(L-lysine)-induced receptor aggregation would be a potential mechanism of kinase activation.

A major difference is seen when comparing activation of receptor phosphorylation by insulin and poly(L-lysine). Poly(L-lysine) markedly stimulates phosphorylation of α -, β - and β_1 -subunits (Figs. 5b and 6), whereas insulin stimulates only β -subunit phosphorylation. This indicates that poly(L-lysine) not only activates the insulin receptor kinase (as measured by the *src*-related peptide) but also acts on the receptor subunits in some manner enabling the α - and β_1 -subunits to be phosphorylated. In the case of α -subunit phosphorylation, it is possible that the acidic domains A-I and A-II (Table 3) also interact

Table 3. Insulin receptor acidic domains that may interact with poly(L-lysine)

The insulin receptor primary structure reported by Ullrich *et al.* [1] 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 (633–645)	ERQAEDSELFELD	13	4 + 2 (45)	1 + 0 (7.7)
A-II (695–707)	ELEESSFRKTFED	13	4 + 1 (38)	1 + 1 (15.4)
A-III (1268–1286)	EENKAPESSELEMEFEDME	19	9 + 1 (53)	0 + 1 (5.3)

with poly(L-lysine), enabling the tyrosine residues to become accessible to phosphorylation by insulin receptor kinase. This mechanism may be similar to that proposed for calmodulin phosphorylation [17]. Monitoring poly(L-lysine)-induced conformational changes of the receptor subunits or calmodulin by titration of accessible tyrosine residues could contribute to further understanding of the mechanism of activation by poly(L-lysine).

Poly(L-lysine) has been used in a variety of biological systems. It has been used as a multivalent intermediary carrier for immunization [31] and to attach cells firmly to glass surfaces [32]. An oligonucleotide-poly(L-lysine) conjugate was introduced for delivery of anti-sense RNA sequences to intact cells [33]. Enzyme activation by basic polycations/proteins have been reported. (i) Poly(L-lysine), poly(D-lysine) and poly(L-ornithine) stimulated phosphorylation *in vitro* of some membrane proteins present in *Xenopus laevis* oocyte membranes, whereas poly(L-arginine) did not [34]. (ii) Poly(L-lysine), histone H1 and protamine markedly stimulated phosphorylase phosphatase in rat and rabbit liver cytosols [35]. (iii) Poly(L-lysine) stimulated a spleen tyrosine kinase-I by 2–3-fold [36]. (iv) A protein serine kinase from membranes of baker's yeast was activated by polypeptides with a high lysine content, such as histone H1 [37]. In contrast, poly(L-lysine) and poly(L-arginine) were potent inhibitors of protein kinase C [38]. In view of this, it is difficult to predict a general mechanism by which poly(L-lysine) affects certain enzymes and proteins. However, the present studies indicate that the mechanism of insulin receptor kinase activation by poly(L-lysine) may be through its interaction with the acidic A-III domain of the β -subunit. The direct interaction between the cytoplasmic kinase domain of the β -subunit and poly(L-lysine) is also consistent with the recent report [39] in which the kinase activity of the purified recombinant cytoplasmic β -subunit domain was shown to be stimulated by poly(L-lysine). Further investigation will be necessary to prove our hypothesis and determine the minimum structural requirement of poly(L-lysine) responsible for the kinase activation.

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