Calcium-dependence of insulin receptor phosphorylation

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Phosphorylation of the insulin receptor of isolated rat adipocytes in response to insulin has been studied. Immunoprecipitation of adipocyte membrane protein demonstrated increased incorporation of ^{32}P after exposure to insulin for 15 min, but this was dependent on the presence of physiological concentrations of Ca^{2+} and Mg^{2+} . Autoradiography of solubilized immunoprecipitated membrane protein after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis revealed that most of the ^{32}P incorporation occurred in a band corresponding to M_r 95 000, which has been identified previously as the β -subunit of the insulin receptor. ^{32}P incorporation was inhibited by 2,4-dinitrophenol and trifluoperazine. It is suggested that insulin-receptor phosphorylation is an energy-requiring process that is Ca^{2+} -dependent and may be modulated by calmodulin. Phosphorylation may proceed independently of glucose transport.

Insulin exerts many of its metabolic effects by binding to a specific receptor in the plasma membrane (Clausen, 1975; Morgan & Whitfield, 1977; Czech, 1981; Jacobs & Cuatrecasas, 1981; Hilf *et al.*, 1981). These effects include phosphorylation and dephosphorylation of membrane-associated and other intracellular proteins (Clausen, 1975; Benjamin & Clayton, 1978; Marchmont & Houslay, 1980; Nilsson *et al.*, 1980). Within 1 min of exposure of cells to insulin, phosphorylation occurs of the 95 000-Da subunit of the insulin receptor from IM-9 lymphocytes (Kasuga *et al.*, 1982a). The time course of this event is consistent with receptor phosphorylation as a step in the sequence of events leading to glucose transport; however, it is possible that phosphorylation and glucose transport may proceed independently. Ca^{2+} binds to the insulin receptor, increasing its affinity for the hormone (Williams & Turtle, 1981a), and is essential for maximal insulin-stimulated glucose transport (Bihler, 1972; Akhtar & Perry, 1979). As well, Ca^{2+} may have a role as a second messenger initiating intracellular events that lead to glucose uptake by cells (Bonne *et al.*, 1978; Czech, 1980). Mg^{2+} is a cofactor for many enzymes of the glycolytic path and may substitute for Ca^{2+} . An increase in intracellular $[\text{Mg}^{2+}]$ occurs as an early response to insulin stimulation (Sanui & Rubin, 1978). Mg^{2+} actively stimulates hexose uptake, protein and DNA synthesis (Rubin & Sanui, 1981), but its role, if

any, in membrane protein phosphorylation is as yet unclear.

Lanthanum and lanthanide ions (e.g. Tb^{3+}) inhibit insulin-stimulated glucose transport in skeletal muscle (Bihler, 1972; Clausen, 1980) and adipocytes (Williams *et al.*, 1982) without reducing insulin binding to its receptor (Williams & Turtle, 1981b). They have been shown to act as competitive inhibitors for Ca^{2+} binding to biological membranes and they do not enter cells (Mikkelsen, 1976). Lanthanides are useful to investigate events that may depend on the movement of Ca^{2+} across plasma membranes in response to insulin binding.

The aim of the present study was to elucidate the role of Ca^{2+} and Mg^{2+} in insulin-receptor phosphorylation using isolated rat adipocytes as an experimental model and to study the relationship between insulin-receptor phosphorylation and glucose transport into the cell.

Materials and methods

2-Deoxy-D-[U- ^{14}C]glucose, [^3H]inulin and sodium [^{32}P]orthophosphate were obtained from Amersham Australia Pty. Ltd., Sydney, Australia; collagenase (type 3) was from Worthington Biochemical Corporation, Freehold, NJ, U.S.A.; bovine serum albumin (RIA grade) was from the Sigma Chemical Co., St. Louis, MO, U.S.A.; silicon oil [viscosity 350 cSt ($3.5 \times 10^{-5} \text{ m}^2 \cdot \text{s}^{-1}$)] was from Dow Corning

Australia Pty. Ltd., Sydney, Australia; sheep anti-(human immunoglobulin G) was from Silenus Laboratories Pty. Ltd., Dandenong, Vic., Australia. Nylon filter cloth (280 μ m) was a gift from G. A. Roseth Pty. Ltd., Lane Cove, N.S.W., Australia. All other reagents were analytical grade. Kodak MR-1 film for autoradiography was obtained from Kodak (Australasia) Pty. Ltd., Annandale, N.S.W., Australia.

Adipocytes were isolated by collagenase digestion from the epididymal fat-pads of non-starved Wistar rats (150–300g) using a modification of the method of Cushman & Wardzala (1980). The medium used for isolation was Krebs–Ringer bicarbonate/Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; 25mM], pH 7.4, containing 1% bovine serum albumin and 0.1mM-glucose. Calcium, magnesium and phosphate were omitted from the medium. Cell counts were performed manually on a haemocytometer. Adipocyte yields varied between digestions, but constant numbers [(4–9) $\times 10^5$ in 500 μ l per tube] were used in each experiment. Cells were pre-incubated in isolation buffer with or without Ca^{2+} and/or Mg^{2+} (final concentrations 2.5mM and 1.2mM respectively). All pre-incubations were performed in the presence of tracer concentrations of [^{32}P]orthophosphate (25mCi/litre). Terbium ions (Tb^{3+}) were added in some incubations with the [^{32}P]orthophosphate and in others immediately before the addition of insulin at the end of the pre-incubation. This eliminated the possibility that Tb^{3+} would precipitate orthophosphate non-specifically. In other experiments pre-incubations contained either 2,4-dinitrophenol (120 μ M and 600 μ M) or trifluoperazine (12 μ M). In those experiments performed to examine the effect of trifluoperazine, this was added on separate occasions for either the entire pre-incubation with [^{32}P]orthophosphate or 10min before the addition of insulin, to avoid the possibility that trifluoperazine would prevent uptake of orthophosphate by adipocytes. Schubart *et al.* (1980) report that trifluoperazine does not prevent uptake of [^{32}P]orthophosphate by intact insulinoma cells.

After 1h, insulin (30ng/ml) was added to the medium and the incubation was continued for a further 15min. The reaction was terminated by the rapid addition of 2ml of iso-osmotic ice-cold assay buffer containing 10mM-NaF, 4mM-EDTA and 20mM-sodium phosphate. Cell viability after incubation was assessed by Acridine Orange exclusion (Lorch & Rentsch, 1969). Cells were separated by centrifuging up through 800 μ l of silicon oil at 2000g and 4°C for 5min in a Beckman J6B centrifuge. The cell pellet was aspirated, washed once, re-centrifuged and again aspirated from the oil layer. The cells were solubilized in 1% Triton X-100 in 300 μ l of assay buffer containing phenylmethanesulphonyl

fluoride (2mM), aprotinin (1000 units/ml) and insulin-receptor antibody [serum B-2, 1:350 dilution (Kasuga *et al.*, 1982a)], 10mM-NaF and the mixture was allowed to reach equilibrium overnight at 4°C. The antibody–insulin-receptor complex was precipitated by incubation with sheep anti-(human immunoglobulin G) at 20°C for 4h, then centrifuged at 3000g for 20min at 4°C. The precipitate was washed once in 0.9% NaCl and then solubilized in electrophoresis buffer [Tris/HCl (40mM), sodium acetate (20mM), EDTA (1mM), 1% sodium dodecyl sulphate and 5% mercaptoethanol] before counting for radioactivity in an LKB-Wallac 81000 liquid-scintillation counter. Radioactivity incorporated into the immunoprecipitable insulin receptor (Table 1) was expressed as a percentage, with the basal radioactivity (no Ca^{2+} , Mg^{2+} or insulin) being assigned a value of 100% in each experiment.

Portions of solubilized immunoprecipitate were analysed by sodium dodecyl sulphate/polyacrylamide gradient (4–30%) gel electrophoresis using markers of known molecular weight (Pharmacia Fine Chemicals, 1980). After electrophoresis, gels were fixed, stained with 0.1% Coomassie Blue R 250 in 25% methanol/10% acetic acid in water at 24V for 30min, then destained under the same conditions. Slab gels were sealed in plastic bags and applied to Kodak MR-1 film for autoradiography for 12 days.

Parallel experiments measuring basal and insulin-stimulated 2-deoxyglucose uptake (Pederson & Gliemann, 1981) were performed on each occasion to ensure cellular responsiveness to insulin. Ca^{2+} (2.5mM), Mg^{2+} (1.2mM) and KH_2PO_4 (1.2mM) were added in each of these experiments. Allowance was made for uptake in the extracellular space by using [^3H]inulin; it did not exceed 0.15% of total uptake in any experiment. Inhibition of insulin-stimulated 2-deoxyglucose transport by 2,4-dinitrophenol (10 μ M–1mM) was measured after pre-incubating cells for 1h with 2,4-dinitrophenol, insulin, Ca^{2+} , Mg^{2+} and P_i . In separate experiments, 2,4-dinitrophenol (10mM) was added to adipocyte suspensions after a 1h pre-incubation with insulin for 10 and 15min before measuring 2-deoxyglucose uptake. The statistical significance of differences was assessed with Student's unpaired *t*-test.

Results

Insulin (30ng/ml), Ca^{2+} (2.5mM) and Mg^{2+} (1.2mM) increased 2-deoxyglucose transport to $283 \pm 12.7\%$ ($n = 36$) of basal values despite the omission of Ca^{2+} and Mg^{2+} from the isolation medium. Immunoprecipitable protein phosphorylation was increased in the presence of 2.5mM- Ca^{2+} and 1.2mM- Mg^{2+} to 142.4% of basal levels and both of these ions were necessary for insulin to achieve the maximal increase of receptor phosphorylation to

226.3% of basal levels. In their absence, insulin increased phosphorylation by only 15.5%.

In experiments where Ca^{2+} or Mg^{2+} were added separately submaximal stimulation of receptor phosphorylation by insulin was observed (164.6% and 144.1% respectively; Table 1). In control experiments for immunoprecipitation using normal human serum (1:350), phosphorylation was $110 \pm 11\%$ for basal and $146 \pm 7\%$ when maximally insulin-stimulated in the presence of Ca^{2+} and Mg^{2+} ($n = 7$). At least 90% of cells were judged viable at the end of each experiment by the criterion of Acridine Orange exclusion (Lorch & Rentsch, 1969).

Although use of normal human serum caused precipitation of some phosphorylated membrane protein (Kasuga *et al.*, 1982*b*) autoradiography of the sodium dodecyl sulphate/polyacrylamide gels demonstrated an absence of radioactivity in the band (M_r 95 000) that was heavily phosphorylated when precipitated by the specific insulin-receptor anti-serum, B-2 (Fig. 1). Insulin-stimulated immunoprecipitable protein phosphorylation was reduced from 226.3% to 127.4% and 49.5% in the presence of 120 μM - and 600 μM -2,4-dinitrophenol respectively (Table 1). In the presence of Tb^{3+} , the membrane protein precipitate contained variable [^{32}P]phosphate radioactivity (149.0% with Tb^{3+} alone; 146.8% with Tb^{3+} and Ca^{2+} , Mg^{2+} and insulin) but sodium dodecyl sulphate/polyacrylamide-gel electrophoresis showed phosphorylation of membrane proteins did not occur under these conditions (Fig. 1). The precipitation may

represent Tb^{3+} complexed with [^{32}P]ATP and [^{32}P]orthophosphate (Dos Remedios, 1981) released during cell solubilization with Triton X-100.

The degree of insulin-receptor phosphorylation together with 2-deoxyglucose uptake by isolated adipocytes in the presence of Ca^{2+} , Mg^{2+} and insulin

Table 1. Phosphorylation of immunoprecipitable membrane protein isolated from rat adipocytes

Results are expressed as percentage increases in phosphorylation, where basal incubations in Krebs-Ringer bicarbonate without Ca^{2+} (2.5 mM), Mg^{2+} (1.2 mM) and insulin (30 ng/ml) are assigned a value of 100%. Results are means $\pm$ s.e.m. for the numbers of observations indicated in parentheses. Additions are as shown. $**P < 0.02$; $***P < 0.01$ versus experiment with Ca^{2+} , Mg^{2+} and insulin.

Experiment	Receptor phosphorylation (%)
Basal	100 (15)
Ca^{2+}	139.4 ± 21.1 (3)
Mg^{2+}	88.7 ± 4.0 (2)
Ca^{2+} , Mg^{2+}	$142.4 \pm 7.4^{**}$ (14)
Insulin	115.5 ± 9.2 (15)
Ca^{2+} , insulin	164.6 ± 28.1 (3)
Mg^{2+} , insulin	144.1 ± 1.4 (2)
Ca^{2+} , Mg^{2+} , insulin	226.3 ± 30.3 (15)
Ca^{2+} , Mg^{2+} , insulin, 2,4-dinitrophenol (120 μM)	$127.4 \pm 12.9^{***}$ (6)
Ca^{2+} , Mg^{2+} , insulin, 2,4-dinitrophenol (600 μM)	$49.5 \pm 20.6^{***}$ (6)

Fig. 1. Autoradiogram (12 days) showing the incorporation of ^{32}P into solubilized insulin receptor from isolated rat adipocytes

The molecular weight of the phosphorylated bands was determined by comparison with proteins of known molecular weight that were run concurrently. Lane (1), basal (no Ca^{2+} , Mg^{2+} or insulin); lane (2), Mg^{2+} ; lane (3), Ca^{2+} ; lane (4), Ca^{2+} , Mg^{2+} ; lane (5), insulin; lane (6), protein standards; lane (7), insulin; lane (8), Ca^{2+} , insulin; lane (9), Mg^{2+} , insulin; lane (10), Ca^{2+} , Mg^{2+} insulin; lane (11), Ca^{2+} , Mg^{2+} , insulin precipitated with normal human serum; lane (12), Ca^{2+} , Mg^{2+} , insulin, Tb^{3+} ; lane (13), protein standards; lane (14), protein standards; lane (15), Ca^{2+} , Mg^{2+} , insulin, trifluoperazine (12 μM).

with either trifluoperazine ($12\mu\text{M}$) or Tb^{3+} (0.5 mM) is shown in Table 2. Although the phosphorylation of the isolated insulin receptor was decreased by trifluoperazine, there was no change in 2-deoxyglucose transport. In a separate experiment, trifluoperazine ($6\mu\text{M}$) added after a 1 h pre-incubation for only 10 min before the addition of insulin, reduced immunoprecipitable protein phosphorylation to $52.4 \pm 3.7\%$ (mean $\pm$ S.E.M.) of the maximum insulin-stimulated level. In the presence of Tb^{3+} , glucose transport was 80% of the maximum insulin-stimulated level, although there was no phosphorylation of membrane proteins (Fig. 1).

Maximal inhibition of 2-deoxyglucose transport by 2,4-dinitrophenol occurred if cells were pre-incubated with $500\mu\text{M}$ -2,4-dinitrophenol for 1 h (Table 3). Addition of 2,4-dinitrophenol for only 10 min before measurement of 2-deoxyglucose trans-

port was sufficient to inhibit 2-deoxyglucose transport maximally (results not shown).

Discussion

Ca^{2+} and Mg^{2+} play an essential role in allowing maximal stimulation of glucose uptake in isolated adipocytes and muscle (Akhtar & Perry, 1979; Czech, 1980). The role of Ca^{2+} in membrane events has been reviewed extensively (Clausen, 1980). It has been proposed that one role for Ca^{2+} may be to activate glucose transport by localized release of a small amount of intracellular Ca^{2+} from the plasma membrane (Kisbah *et al.*, 1975). A non-specific increase of intracellular Ca^{2+} induced by the ionophore A23187 (Bonne *et al.*, 1978) or by inhibition of the plasma-membrane $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent ATPase by Tb^{3+} (Williams *et al.*, 1982) is insufficient to activate this effect. In the presence of physiological concentrations of Ca^{2+} and Mg^{2+} , phosphorylation of the immunoprecipitable insulin receptor is increased significantly. Both Ca^{2+} and Mg^{2+} are required for further stimulation of receptor phosphorylation by insulin. It was assumed that intracellular $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ had equilibrated with externally added $[\text{P}^{32}]\text{orthophosphate}$ after the 1 h pre-incubation (Denton *et al.*, 1981).

Tb^{3+} may replace Ca^{2+} on protein-binding sites (Martin & Richardson, 1979) and like Ca^{2+} it increases insulin binding to the receptor (Williams & Turtle, 1981b). In the presence of Tb^{3+} , there is no phosphorylation of membrane proteins precipitated by anti-(insulin receptor) antibody B-2. Tb^{3+} prevents the increment in insulin-receptor phosphorylation induced by insulin. Thus Ca^{2+} is necessary for insulin-receptor phosphorylation. There may be local Ca^{2+} movements (Clausen, 1980) or the specific activation of a Ca^{2+} -dependent kinase as a consequence of insulin binding to its receptor. Further study is required to identify this putative kinase.

Mg^{2+} is required as cofactor by many glycolytic enzymes (e.g. hexokinase, phosphofructokinase), and Mg^{2+} acts in association with ATP in several transphosphorylation reactions. Experiments in cultured chick fibroblasts (Sanui & Rubin, 1978) have demonstrated that a rise in intracellular $[\text{Mg}^{2+}]$ occurs within 10 min of exposure of these cells to insulin. In addition externally bound Mg^{2+} and Ca^{2+} are released into the medium by insulin in a dose-dependent fashion.

The data demonstrate that, although maximal phosphorylation of the insulin receptor occurred in the presence of insulin, Ca^{2+} and Mg^{2+} , significant phosphorylation of the M_r -95 000 band still occurred if Mg^{2+} was omitted from the pre-incubation. Autoradiography of the sodium dodecyl sulphate/polyacrylamide gels showed that incorporation of

Table 2. *Insulin-receptor phosphorylation and 2-deoxyglucose transport in isolated rat adipocytes stimulated with insulin in the presence of trifluoperazine and Tb^{3+}*

Results are expressed as percentages of the maximum phosphate incorporation and 2-deoxyglucose transport obtained with isolated rat adipocytes in the presence of Ca^{2+} (2.5 mM), Mg^{2+} (1.2 mM) and insulin (30 ng/ml). * indicates no receptor phosphorylation was detected in the presence of Tb^{3+} (Fig. 1). All results are means $\pm$ S.E.M. for the numbers of observations indicated in parentheses.

Experiment	Receptor phosphorylation (%)	2-Deoxyglucose transport (%)
Ca^{2+} , Mg^{2+} , insulin	100 (16)	100 (16)
Ca^{2+} , Mg^{2+} , insulin, Tb^{3+} (0.5 mM)	0* (15)	80.0 ± 7.3 (12)
Ca^{2+} , Mg^{2+} , trifluoperazine ($12\mu\text{M}$)	21.3 ± 2.9 (6)	99.3 ± 2.8 (8)

Table 3. *Inhibition of insulin-stimulated 2-deoxyglucose transport by 2,4-dinitrophenol*

All experiments were performed in the presence of Ca^{2+} (2.5 mM), Mg^{2+} (1.2 mM) and P_i (1.2 mM). Insulin concentration was 30 ng/ml . Results are expressed as percentages of the maximum insulin-stimulated 2-deoxyglucose transport. Values are means $\pm$ S.E.M. for the numbers of observations shown in parentheses.

Experiment	2-Deoxyglucose transport (%)
Basal	43.2 ± 2.1 (4)
Insulin	100 (4)
Insulin, 2,4-dinitrophenol ($10\mu\text{M}$)	97.9 ± 3.5 (4)
Insulin, 2,4-dinitrophenol ($50\mu\text{M}$)	99.8 ± 5.2 (4)
Insulin, 2,4-dinitrophenol ($100\mu\text{M}$)	75.4 ± 0.8 (3)
Insulin, 2,4-dinitrophenol ($500\mu\text{M}$)	8.9 ± 0.9 (4)
Insulin, 2,4-dinitrophenol ($1000\mu\text{M}$)	9.4 ± 0.8 (4)

[^{32}P]phosphate into the M_r -95 000 band was greater in the presence of Ca^{2+} and insulin than in experiments performed with Mg^{2+} and insulin (Fig. 1, Table 4). It seems probable that Ca^{2+} is the more important of the two cations in this process.

Incorporation of ^{32}P into the solubilized membrane proteins or membrane lipids does not occur after incubation with Tb^{3+} .

Reduction of receptor phosphorylation in the presence of 2,4-dinitrophenol suggests that transphosphorylation from P_i is not the source of phosphate groups in this transformation and that ATP or GTP is the probable substrate for the reaction (Czech, 1980). It is probable that the action of 2,4-dinitrophenol was to prevent utilization of intracellular ATP or GTP rather than preventing adipocyte uptake of [^{32}P] P_i , since addition of 2,4-dinitrophenol for 10 min after a 1 h preincubation of cells with insulin, Ca^{2+} , Mg^{2+} and P_i is sufficient to inhibit maximally 2-deoxyglucose transport. Trifluoperazine inhibited insulin-receptor phosphorylation, suggesting that calmodulin may mediate insulin-receptor phosphorylation. Despite this inhibition of phosphorylation after insulin stimulation, glucose transport was unaffected (Table 2).

Insulin-stimulated phosphorylation was decreased in the presence of Tb^{3+} with only minor alterations in glucose transport. Of course the presence of trifluoperazine or Tb^{3+} may so alter membrane or receptor structure as to prevent receptor phosphorylation by a kinase [which may be in the receptor itself or loosely attached (Kasuga *et al.*, 1982b)]. Therefore receptor phosphorylation may not be essential for insulin stimulation of glucose transport. Alternatively a minor alteration in receptor phosphorylation states (<10%) may be necessary for maximum stimulation of glucose transport.

It appears that ATP, Ca^{2+} and possibly calmodulin are essential for insulin receptor phosphorylation, but phosphorylation of the receptor may not be a prerequisite for insulin-stimulated glucose transport.

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Table 4. *Densitometry of the autoradiogram shown in Fig. 1*

Values represent the area under the curve for phosphorylation in the band corresponding to M_r 95 000. Densitometry was performed on a CAMAG Electrophoresis Scanner (CAMAG, Muttens, Switzerland). Areas were calculated by using a SHIMADZU CR1A Integrator Plotter.

Lane	Experiment	Area (arbitrary units)
1	Basal	299
4	Ca^{2+} , Mg^{2+}	487
5	Insulin	214
8	Ca^{2+} , insulin	651
9	Mg^{2+} , insulin	582
10	Ca^{2+} , Mg^{2+} , insulin	872
12	Ca^{2+} , Mg^{2+} , insulin, Tb^{3+}	0
15	Ca^{2+} , Mg^{2+} , insulin, trifluoperazine	0

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