Insulin-induced myosin light-chain phosphorylation during receptor capping in IM-9 human B-lymphoblasts

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We have examined further the interaction between insulin surface receptors and the cytoskeleton of IM-9 human lymphoblasts. Using immunocytochemical techniques, we determined that actin, myosin, calmodulin and myosin light-chain kinase (MLCK) are all accumulated directly underneath insulin-receptor caps. In addition, we have now established that the concentration of intracellular Ca^{2+} (as measured by fura-2 fluorescence) increases just before insulin-induced receptor capping. Most importantly, we found that the binding of insulin to its receptor induces phosphorylation of myosin light chain *in vivo*. Furthermore, a number of drugs known to abolish the activation properties of calmodulin, such as trifluoperazine (TFP) or W-7, strongly inhibit insulin-receptor capping and myosin light-chain phosphorylation. These data imply that an actomyosin cytoskeletal contraction, regulated by Ca^{2+} /calmodulin and MLCK, is involved in insulin-receptor capping. Biochemical analysis *in vitro* has revealed that IM-9 insulin receptors are physically associated with actin and myosin; and most interestingly, the binding of insulin to the isolated insulin-receptor/cytoskeletal complex significantly enhances the phosphorylation of the 20 kDa myosin light chain. This insulin-induced phosphorylation is inhibited by calmodulin antagonists (e.g. TFP and W-7), suggesting that the phosphorylation is catalysed by MLCK. Together, these results strongly suggest that MLCK-mediated myosin light-chain phosphorylation plays an important role in regulating the membrane-associated actomyosin contraction required for the collection of insulin receptors into caps.

INTRODUCTION

Peptide hormones such as insulin regulate a variety of important cellular functions (e.g. glucose uptake, glycogenesis and lipogenesis) [1]. All insulin-induced cellular activities are known to be initiated by the binding of insulin to its specific receptor on the cell surface. [1]. The insulin receptor has been characterized as a glycoprotein composed of two subunits (α -subunit of 130 kDa, and β -subunit of 95 kDa) joined together by disulphide bonds [2,3]. The 130 kDa subunit is the insulin-binding site, and the 95 kDa subunit has tyrosine kinase activity. The regulatory events involved in insulin-mediated cellular activation [e.g. insulin + receptor (130 kDa protein) $\rightarrow$ the activation of tyrosine kinase (95 kDa protein)] and subsequent cellular responses remain unresolved [4–7].

Upon binding of insulin, the surface receptors are induced to form small clusters ('patches') and/or large aggregates ('caps') [8–10]. The results of previous double-immunofluorescence experiments indicate that actomyosin, actin-associated proteins and myosin light-chain kinase (MLCK) are all accumulated beneath the insulin-induced receptor patch and cap structures [11]. Furthermore, a number of calmodulin inhibitors, such as trifluoperazine (TFP), W-5, W-7 and W-12, have been shown to inhibit both calmodulin-dependent MLCK activity [12] and insulin-induced cap formation [11]. Therefore it has been postulated that insulin-induced receptor movement is regulated by a calmodulin-dependent MLCK [11]. However, MLCK-mediated myosin light-chain phosphorylation during insulin capping has not been established at present.

Phosphorylation of myosin light chain has been shown to enhance the actin-activated Mg^{2+} -activated ATPase of myosin [2,13]. This process may be a central event in the overall regulation of contractility in both smooth-muscle and non-muscle cells [1]. Phosphorylated myosin light chain has been shown to become tightly associated with the plasma membranes of platelets during their activation [14]. Recently, we have found in mouse T-lymphoma cells that myosin light chain is not only preferentially phosphorylated but also selectively associated with the plasma-membrane receptor during surface patching/capping [15]. Furthermore, we have demonstrated that a Ca^{2+} /calmodulin-dependent MLCK activity is always present in a lymphoma membrane-cytoskeleton complex and that this activity is intimately involved in receptor aggregation [16]. Together, these pieces of evidence strongly support the hypothesis that myosin light-chain phosphorylation is critically important in the regulation of surface-receptor motility.

In order to examine the apparent structural interactions occurring between insulin surface receptors and the underlying cytoskeletal components, we have chosen the IM-9 human lymphoblast cell line as a relevant model system for this study. We consider that this cell line is ideal for this type of study, because (1) the insulin receptor from IM-9 human lymphoblasts has been biochemically characterized by a number of laboratories [17–19], and (2) the IM-9 cell line displays insulin-induced receptor capping which appears to be regulated by MLCK [11].

In the present study we initially verified and extended our previous findings that insulin-receptor capping is

Abbreviations used: TFP, trifluoperazine; MLCK, myosin light-chain kinase; DSS, disuccinimidyl suberate; DPBS, Dulbecco's phosphate-buffered saline; PMSF, phenylmethanesulphonyl fluoride; PAGE, polyacrylamide-gel electrophoresis.

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a Ca^{2+} /calmodulin/MLCK-related process, and then employed a variety of complementary techniques (i.e. fura-2 fluorescence measurement, chemical cross-linking, non-ionic-detergent extraction, immunoprecipitation and gel electrophoresis) to explore mechanisms involved in the regulation of insulin-induced receptor capping in IM-9 human lymphoblasts.

MATERIALS AND METHODS

Cells

Human B lymphoblasts (the IM-9 cell line) were obtained from the American Type Culture Collection and grown in RPMI 1640 medium (GIBCO), supplemented with 10% (v/v) fetal-bovine serum, 25 mM-Hepes (Sigma Chemical Co.), 2 mM-L-glutamine, 100 units of penicillin/ml and 100 μg of streptomycin/ml at 37 °C in CO_2 /air (1:19) [20].

Measurement of intracellular Ca^{2+}

IM-9 cells grown as described above (2×10^7 cells/ml) were loaded with 5 mM-fura-2 (Calbiochem) for 1 h at 25 °C in 145 mM-NaCl/5 mM-KCl/0.1 mM- MgCl_2 /1 mM- CaCl_2 /5.5 mM-glucose/15 mM-Hepes, pH 7.3. Cells were washed three times with this buffer and diluted to a final concentration of 1×10^7 cells/ml for fluorimetry in an Hitachi MPF-2A fluorescence spectrophotometer with a Perkin-Elmer 150 Xenon power supply. A 2 ml sample of cells was placed in each cuvette and treated with insulin (1 μM) while fluorescence was monitored at 500 nm emission with excitation wavelengths of both 340 nm and 380 nm. Ca^{2+} concentration was determined by using the equation

$$[\text{Ca}^{2+}] = K_d(R - R_{\min.}/R_{\max.} - R)(S_{f2}/S_{b2}),$$

with $K_d = 135$, $S_{f2}/S_{b2} = \text{constant}$ calculated by Ca^{2+} calibration for each run, and $R = \text{fluorescence ratio } F_{340}/F_{380}$ as described previously [21].

Induction of insulin-receptor capping

Cells were harvested from growth medium, washed three times, and resuspended in buffer containing 100 mM-Hepes, 120 mM-NaCl, 1.2 mM- MgSO_4 , 2.5 mM-KCl, 1 mM-EDTA, 10 mM-glucose and 1.5 mM-sodium acetate, pH 7.8 [22]. Approx. 10^8 cells/ml were incubated in this medium plus 10 nM unlabelled insulin (generously given by Eli Lilly and Co., Indianapolis, IN, U.S.A.; lot no. 615-2H2-300) at 15 °C for 1 h. After unbound insulin was washed off at 4 °C, cells were subsequently warmed to room temperature for 20 min to allow receptors to cap. In order to observe insulin-receptor capping, cells were then fixed in 0.5% paraformaldehyde in Dulbecco's phosphate-buffered saline (DPBS) (pH 7.5) for 20 min at 0 °C, washed in ice-cold DPBS/0.1 M-glycine, and labelled by the following procedure. First, normal mouse immunoglobulin was used to pre-bind any non-specific Ig binding sites, such as Fc receptors; this was followed by the incubation of human serum containing antibodies directed against insulin receptors (from patient Bd; generously given by Dr. Guenther Boden, Temple Medical School, Philadelphia, PA, U.S.A.), plus fluorescein-conjugated goat anti-human immunoglobulin (Cooper Biomedical) [23]. As a control, insulin-capped cells were fixed and stained with non-immune human serum (Cooper Biomedical). For counting purposes, insulin capping was scored positive when the fluorescence

membrane label concentrated into one half or less of the cell. A minimum of 100–200 cells was examined per experiment.

Double-immunofluorescence microscopy

For localization of intracellular proteins, fluorescein-labelled insulin-capped cells were mounted on poly-L-lysine-coated slides. Cells were rendered permeable by treatment with 0.5% Triton X-100 (in DPBS/glycine for 15 min at 25 °C), followed by 95% (v/v) ethanol for 5 min. Permeabilized cells were labelled with sheep anti-calmodulin serum (provided by Dr. Michael Welsh, University of Michigan, Ann Arbor, MI, U.S.A.), rabbit anti-(human uterus smooth-muscle myosin light chain) serum (made by L. Y. W. B.) [15], or rabbit anti-MLCK serum (generously given by Dr. Anthony Means, Baylor College, Houston, TX, U.S.A.), followed by rabbit anti-sheep antibody (IgG fraction) or goat anti-rabbit antibody (IgG fraction) (Cooper Medical) respectively, conjugated with rhodamine sulphonyl chloride by the method of Brandtzaeg [23]. Samples were post-fixed before viewing.

All fluorescent samples were mounted for viewing with 0.01% phenylenediamine in 10% DPBS/90% glycerol medium [24]. Fluorescence observations were made with a Zeiss photo-microscope equipped with epi-illumination. The fluorescein fluorescence was excited with an Osram XBO 75 W bulb via a CZ 487710 filter, whereas rhodamine fluorescence was excited with a CZ 487714 filter. All samples were photographed using a $\times 63$ oil-immersion lens and Kodak Tri-X film.

Effects of calmodulin-binding drugs on insulin-receptor capping

Before the addition of insulin, cells were pretreated with one of the following drugs: 20 μM -trifluoperazine (TFP; Smith, Kline and French, Philadelphia, PA, U.S.A.), 20 μM -W-5, 20 μM -W-7, 20 μM -W-12 (Diversified Biotech, Boston, MA, U.S.A.) for 30 min at 0 °C. These drugs were maintained at these concentrations throughout insulin incubation, washing, and capping as previously described. Fixation and anti-(insulin-receptor) antibody labelling were performed as described.

Gel electrophoresis and radioactivity analysis

SDS/PAGE was conducted in an exponential polyacrylamide gradient (6.0–17%) and the discontinuous buffer system of Laemmli [25]. Samples were dissolved in a buffer containing 2% (w/v) SDS, 0.1 M-2-mercaptoethanol, 0.003% Bromophenol Blue, 20 mM-Tris/HCl, 12.5% (v/v) glycerol, pH 6.8, and then heated for 2 min at 100 °C. Electrophoresis was carried out at constant current of 3 mA at room temperature overnight.

Glycerol/urea/PAGE was conducted in the buffer system 20 mM-Tris/glycine, pH 8.6, as described by Perrie & Perry [26]. Samples were dissolved in a buffer consisting of 8 M-urea, 33 mM-Tris/glycine, pH 8.6, 0.17 mM-EDTA, 10 mM-dithiothreitol and 0.003% Bromophenol Blue, and were electrophoresed in gels composed of 10% acrylamide, 1.5% bisacrylamide and 40% glycerol, at 6 W at room temperature. Protein standards such as phosphorylated and unphosphorylated smooth-muscle myosin light chain (provided by Dr. W. G. L. Kerrick, University of Miami, Miami, FL, U.S.A.) were used in this analysis. The polypeptide-banding pattern was made visible by staining with

Coomassie Blue. For quantification of radioactively labelled proteins, both SDS/PAGE and glycerol/urea/PAGE gels were vacuum dried, and exposed to Kodak X-ray (X-Omat XAR-5) film at -70°C .

¹²⁵I-insulin binding

In order to label the insulin-binding sites on IM-9 cell surface, cells were incubated with 0.5 nM -¹²⁵I-insulin (ICN) in insulin-binding buffer (containing 100 mM -Hepes, 120 mM -NaCl, 1.2 mM -MgSO₄, 2.5 mM -KCl, 1 mM -EDTA, 10 mM -glucose, 1.5 mM -sodium acetate, pH 7.8) [22] at 15°C for 1 h and washed extensively at 4°C . Cross-linking of insulin to its receptor was accomplished with disuccinimidyl suberate (DSS; Pierce Chemical Co.) with 15 min on ice. This reaction was terminated by the addition of 100 mM -Tris/ 10 mM -EDTA in DPBS [27]. ¹²⁵I-insulin binding was determined with an LKB Gamma counter.

Membrane isolation

A crude membrane preparation (with or without ¹²⁵I-insulin treatment) was isolated by the method described by Lang *et al.* [22], with slight modifications. IM-9 cells were homogenized at 4°C with a Potter-Elvehjem homogenizer. Homogenization medium consisted of 40% sucrose in 50 mM -Hepes-buffered Earle's salts, pH 7.5, containing 1 mM -PMSF, $4\text{ }\mu\text{g}$ of aprotinin/ml and $10\text{ }\mu\text{g}$ of leupeptin/ml. The homogenate was centrifuged at 600 g_{av} for 10 min, and the resulting supernatant was centrifuged at $43000\text{ g}_{\text{av}}$ for 30 min, yielding the crude membrane pellet.

Non-ionic-detergent extraction

The IM-9 crude membranes (with or without ¹²⁵I-insulin treatment) were washed twice by resuspension in homogenization medium minus sucrose, then finally resuspended in 1 mg of protein/ml in extraction buffer, consisting of 150 mM -NaCl, 50 mM -Hepes (pH 7.5), 1 mM -PMSF, $1\text{ }\mu\text{g}$ of aprotinin/ml, $1\text{ }\mu\text{g}$ of leupeptin/ml, 100 mM -Na₄P₂O₇, 50 mM -NaF and 1% (v/v) Triton X-100. This radioactively labelled material was extracted at 4°C for 1 h with constant stirring and then centrifuged at $110000\text{ g}_{\text{av}}$ for 1 h. The $110000\text{ g}_{\text{av}}$ -pellet fraction was discarded and the supernatant fraction (containing insulin-binding sites) was collected for further analysis.

Immunobinding procedures

IM-9 crude membranes (with or without ¹²⁵I-insulin treatment) were extracted by 1% Triton X-100 and centrifuged at $110000\text{ g}_{\text{av}}$ for 1 h as described above. The supernatant fractions from the $110000\text{ g}_{\text{av}}$ centrifugation were used for immunobinding assays. To prepare anti-(insulin receptor)-antibody-conjugated gel beads, human anti-(insulin receptor) antiserum was added to glutaraldehyde-activated Ultrogel beads (ACA 22; LKB) and incubated at 4°C for 6 h. A small column of these conjugated immunobeads was prepared, and samples of Triton X-100-solubilized plasma membrane were passed through. This was followed by extensive washing with 0.1% Triton X-100. As a control for non-specific binding, a column was prepared with pre-absorbed anti-(insulin receptor)-conjugated gel beads. Cellular materials bound to the anti-(insulin receptor)-conjugated beads (designated as insulin receptor-cytoskeleton complexes) were subsequently solubilized by SDS and analysed by SDS/PAGE and autoradiography as described above.

Immunoblotting and immunoprecipitation of myosin light chain

For immunoblotting, the IM-9 insulin-receptor-cytoskeleton complex (prepared by the procedures described above) were analysed by SDS/PAGE. Subsequently they were transferred to nitrocellulose sheets, followed by incubation with rabbit anti-(myosin light chain) serum or pre-absorbed sera (anti-myosin-free sera) [15] and ¹²⁵I-labelled goat anti-rabbit IgG or Protein A. The nitrocellulose was washed extensively, dried, and analysed by autoradiography as described previously [28]. For immunoprecipitation, the following experiments were carried out. (1) Triton-X-100-solubilized IM-9 plasma membranes were incubated with rabbit anti-(human smooth-muscle myosin light chain) serum ($10\text{ }\mu\text{g/ml}$), or with pre-absorbed antisera ($10\text{ }\mu\text{g/ml}$) (as a control). (2) Unlabelled smooth-muscle and mouse T-lymphoma myosin were incubated with rabbit anti-(smooth-muscle myosin light chain) serum ($10\text{ }\mu\text{g/ml}$), or with pre-absorbed antisera (as a control), respectively, for 1 h at 0°C . These were followed by the secondary antibody, goat anti-rabbit immunoglobulin ($100\text{ }\mu\text{g/ml}$), overnight at 0°C . Immunoprecipitates were recovered by centrifugation at 800 g for 10 min and analysed by SDS/PAGE and autoradiography as described above.

Measurement of insulin-induced myosin light-chain phosphorylation

In order to label total cellular phosphoproteins *in vivo*, cells were washed twice in phosphate-free RPMI medium and incubated with 1 mCi of H₃³²PO₄ at 37°C for 1 h. Metabolically ³²P-labelled cells were washed extensively in the phosphate-free RPMI medium and incubated in a buffer containing 100 mM -Hepes, 120 mM -NaCl, 1.2 mM -MgSO₄, 2.5 mM -KCl, 1 mM -EDTA, 10 mM -glucose and 1.5 mM -sodium acetate, pH 7.8 [22], plus insulin (10 nM) at 15°C for 1 h. After unbound insulin was washed off at 4°C , cells were subsequently warmed to room temperature for various times (5, 10, 15, 20, 25 and 30 min) to allow insulin-receptor capping to occur. Cells with the maximal capped structures (incubation for approx. 15–20 min at room temperature with insulin) were used to analyse their insulin-induced myosin light-chain phosphorylation. Plasma membranes from ³²P-labelled cells were isolated by the procedures described above, followed by treatment with 1% Triton X-100. These detergent-solubilized materials were immunoprecipitated with rabbit anti-(myosin light-chain) antibody, followed by glycerol/urea/PAGE (for separating phosphorylated and unphosphorylated species of myosin light chains) as described above. The bands corresponding to phosphorylated myosin light chain were cut out, and the phosphate content (expressed in mol) was determined. The protein contents of these phosphorylated myosin light-chain bands were determined by scanning densitometry of a Coomassie-Blue-stained gel with protein standard, and this value was converted into mol of myosin light chain.

Assay of MLCK activity

The MLCK assay procedures used in this study were generally the same as those described previously [16]. The reaction mixture contained crude enzyme preparation [such as IM-9 plasma membranes of insulin-receptor-cytoskeleton complex fractions isolated from

Triton-X-100-solubilized plasma membranes, which were incubated with anti-(insulin receptor) antibody-conjugated immunobeads as described above], 20 mM-KCl, 1 mM-magnesium acetate, 0.1 mM- Ca^{2+} , with or without the addition of insulin (10 nM), 5 μM -calmodulin and 1 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (100–200 c.p.m./pmol). As controls, calmodulin was eliminated and 0.1 M- Ca^{2+} (pCa = 4.0, high Ca^{2+}) was replaced by 10 nM- Ca^{2+} (pCa = 8.0, low Ca^{2+}) in the reaction mixture. In some cases, the enzyme assays were carried out in the presence of 20 μM -TFP or 20 μM -W-7. Reactions were initiated by the addition of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (ICN Chemical and Radioisotope Division, Irvine, CA, U.S.A.) and incubated for 5 min at 30 °C. Myosin light-chain phosphorylation was assayed by the following three steps: (a) spotting 0.005 ml of reaction mixture on Whatman 31 ET chromatography paper, followed by precipitation with 10% trichloroacetic acid; (b) immunoprecipitating the reaction mixture with rabbit anti-(myosin light-chain) antibody, analysing by SDS/PAGE, cutting out and determining the radioactivity contents of the bands corresponding to 20 kDa myosin light chain; and (c) analysing the reaction mixture by glycerol/urea/PAGE (for separating phosphorylated and unphosphorylated species of myosin light chains [26]. The bands corresponding to phosphorylated myosin light chains were cut out and the phosphate content (expressed in mol) was determined. The protein contents of these phosphorylated myosin light-chain bands were determined by scanning densitometry of a Coomassie-blue-stained gel with protein standards, and this value was converted into mol of myosin light chain. The results of MLCK assay (Tables 1 and 2) were obtained by glycerol/urea/PAGE. This method was shown to be very reproducible, as evidenced by the small standard deviations obtained in Table 2.

RESULTS

Measurement of intracellular free Ca^{2+} concentration after binding of insulin

Ca^{2+} ions are believed to control a variety of cellular processes [29]. Recently, the fluorescent Ca^{2+} indicator fura-2 has been used to measure intracellular Ca^{2+} concentrations in various cell types during cell activation [21,30]. In the present study we measured the fluorescence of insulin-treated IM-9 cells loaded with fura-2, in order to determine the intracellular Ca^{2+} concentration during insulin receptor capping. As shown in Fig. 1, the fluorescence signal began to increase between 2 and 5 min after the addition of insulin to fura-2-loaded cells. At about 15 min after addition of insulin, the fura-2 fluorescence reached a maximum, corresponding to a free Ca^{2+} concentration of approx. 170 nM. Although the coincidence of Ca^{2+} activity and capping during insulin treatment did not establish a cause/effect relationship of these two cellular events, the fact that the rise in intracellular Ca^{2+} (Fig. 1a) just preceded the peak time for insulin-induced capping (Fig. 1b) suggests that Ca^{2+} may be involved in the initial events required for cap formation.

Intracellular localization of calmodulin, MLCK, actin and myosin molecules during insulin-induced capping

Using double immunofluorescent staining, we found that intracellular calmodulin (Fig. 2b) was accumulated underneath the insulin-induced capped structure (Fig.

2a). Furthermore, MLCK and actin and myosin molecules (Figs. 2d and 2f) also displayed membrane-associated localization beneath the insulin-induced caps (Figs. 2c and 2e). The differences between calmodulin (Figs. 2b) and MLCK (Fig. 2d) labelling patterns may be due to their differential responses towards insulin treatment. As controls, insulin-capped cells (Fig. 2g) were labelled with non-immune serum (Fig. 2h). The control samples showed no intracellular fluorescent staining (Fig. 2h), which confirms the specificity of this immunocytochemical staining technique. Therefore, these data show that the elements for the involvement of a Ca^{2+} /calmodulin/MLCK-dependent process in insulin-induced surface receptor movement are present in the cap structure.

Effect of calmodulin-binding drugs on insulin-receptor capping and myosin light-chain phosphorylation

Phosphorylation of myosin light chain has been shown to enhance the actin-activated Mg-ATPase of myosin [2,13]. This process may be a central event in the overall regulation of muscle contraction [1] and surface receptor capping [15,16,31]. In the present study, we found that myosin light-chain phosphorylation was greatly increased during insulin-receptor capping (Table 1a). Our preliminary data indicate that phosphorylation of myosin light chain reaches a maximum approx. 2–3 min earlier than insulin-receptor cap formation (results not shown). In addition, both TFP and W-7, which are known to abolish the activation properties of calmodulin, strongly inhibited insulin-induced myosin light-chain phosphorylation and receptor capping (Table 1b). These data suggest that insulin-induced receptor capping and myosin light-chain phosphorylation are both regulated by a calmodulin-dependent process.

Physical association of insulin receptors with the cytoskeleton

In order to identify the insulin-binding sites on the IM-9 cell surface, we labelled the cells with ^{125}I -insulin. However, since the insulin-receptor association is relatively weak, a chemical cross-linking procedure using the bifunctional cross-linking reagent DSS was chosen. To achieve the highest extent of cross-linking with minimal amount of non-specific binding, 10 μM -DSS was used to attach ^{125}I -insulin covalently to its receptor (Fig. 3a). Prebinding with unlabelled insulin at 10 nM successfully inhibited the subsequent binding of ^{125}I -insulin to the cells (Fig. 3b), indicating that the ^{125}I -insulin binding to its receptors is specific.

To analyse further the association between insulin receptors and their underlying cytoskeletal components, Triton-X-100-solubilized IM-9 plasma membrane was incubated with an anti-(insulin receptor)-antibody-conjugated bead column. This method allows one to isolate the insulin-receptor-cytoskeleton complex. Our results showed that this immunobinding assay selectively co-isolated the insulin receptors (as indicated by the presence of 130 kDa ^{125}I -insulin-binding sites) (Fig. 4a) and actin/myosin (200 kDa heavy chain and associated 20 kDa light chain) (Fig. 4b). The specificity of these immunobead columns was established by the fact that a column made with antiserum pre-absorbed with insulin receptors bound neither insulin receptors nor any of the other cytoskeletal components (Fig. 4c).

Using a specific anti-(smooth-muscle myosin light-

Fig. 1. Insulin-stimulated increase in intracellular Ca^{2+} concentration and insulin-receptor capping

After the addition of insulin ($1 \mu M$) (arrow) to fura-2-loaded IM-9 cultured lymphoblastoid cells, the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) gradually rose to a maximum of approx. 170 nM by 15 min (a). This initial increase in Ca^{2+} preceded insulin-induced insulin receptor capping in these cells (b), suggesting that Ca^{2+} may be involved in the initial step required for insulin-receptor cap formation.

Fig. 2. Simultaneous localization of insulin receptors and intracellular cytoskeletal proteins

Double immunofluorescence staining of IM-9 cells was performed to localize surface insulin-receptor cap structures (a, c, e) and the intracellular cytoskeleton-associated proteins calmodulin (b), MLCK (d) and myosin (f) [the same result (not shown) was observed for intracellular localization of actin]; insulin caps in panels (a), (c) and (e) are from the same cells shown in the corresponding panels (b), (d) and (f) respectively. As controls, capped cells (g) stained with non-immune serum (h) showed a negligible non-specific fluorescence staining.

chain) antibody and standard immunoblotting technique, we have further verified that the 20 kDa protein in the insulin-receptor-cytoskeleton complex is the myosin light chain (Fig. 4d). The specificity of this immunoreagent has been established by the facts that (a) anti-(smooth-muscle myosin light-chain) antibody precipi-

tated myosin light chains along with myosin heavy chains in lymphoma cells and smooth-muscle cells (Figs. 4f and 4g) by immunoprecipitation, and (b) an anti-myosin-light-chain-free serum (i.e. pre-absorbed with purified smooth-muscle myosin light chain) precipitated neither myosin nor any of the other proteins (Fig. 4h).

Table 1. Correlation between insulin-induced capping and myosin light-chain phosphorylation**(a) Insulin-induced myosin light-chain phosphorylation**

Conditions	Phosphorylation (mol of P_i /mol of myosin light chain)
Untreated cells	0.105 ± 0.11
Insulin-induced capped cells	0.788 ± 0.15

(b) Effect of calmodulin-binding drugs on insulin-induced receptor capping and myosin light-chain phosphorylation*

Drug treatment	Insulin-induced receptor capping (% of control)	Insulin-induced 20 kDa myosin light-chain phosphorylation (% of control)
Control (no treatment)	100	100
TFP (20 μ M)	15	12
W-7 (20 μ M)	20	18

* Assays for insulin-induced capping and myosin light-chain phosphorylation were described in the Materials and methods section. Data given represent the capping percentage and myosin light-chain phosphorylation from four separate experiments with S.D. $< \pm 5\%$.

Presence of MLCK activity in the insulin-receptor-cytoskeleton complex

Experiments *in vivo* showed that insulin-induced myosin light-chain phosphorylation was stimulated during insulin-receptor capping (Table 1). Further analysis *in vitro* indicated that the insulin-receptor-cytoskeletal fraction contained a Ca^{2+} /calmodulin-mediated (trifluoperazine-, TFP- and W-7-sensitive) kinase activity (Table 2a) which was capable of phosphorylating the endogenous 20 kDa IM-9 myosin light chain (Fig. 4e). This kinase is most probably MLCK,

Fig. 3. Cross-linking of insulin to IM-9 cells

Various concentrations of the cross-linking reagent DSS were used to attach insulin covalently to its receptors on IM-9 cells. With increasing concentrations of DSS, increasing amounts of ^{125}I -insulin were found associated with the cells (a). In the presence of an excess of unlabelled insulin, minimal ^{125}I -insulin was associated with these cells (b), indicating the specificity of insulin binding to its receptor.

since it is both Ca^{2+} - and calmodulin-dependent (Table 2). Most importantly, we found that during the binding of insulin to its receptor *in vitro*, myosin light-chain phosphorylation proceeds from a low degree of phosphorylation to the fully phosphorylated form (Table 2b). We also detected the phosphorylation of a 95 kDa polypeptide in this insulin-receptor-cytoskeletal complex (Fig. 4e). This protein is probably the β -subunit of insulin receptor, which is known to be auto-phosphorylated by its associated tyrosine kinase activity [4,6]. Furthermore, phosphorylation of this 95 kDa protein was neither dependent on Ca^{2+} /calmodulin nor sensitive to calmodulin-binding drugs (results not shown).

Table 2. Assay *in vitro* for MLCK activity in the insulin-receptor-cytoskeletal complex

The ability of IM-9 MLCK to phosphorylate the endogenous IM-9 myosin light chain was measured by the procedures described in the Materials and methods section. Each value represents the average of three experiments, with S.D. $< \pm 5\%$.

Treatment	Condition	MLCK activity (mol of P_i /mol of myosin light chain)
(a) No insulin added	pCa 8.0	0.127
	pCa 4.0 + calmodulin	0.360
	pCa 4.0 + calmodulin + TFP	0.120
	pCa 4.0 + calmodulin + W-7	0.102
(b) Insulin (10 nM) added	pCa 8.0	0.134
	pCa 4.0 + calmodulin	0.879
	pCa 4.0 + calmodulin + TFP	0.121
	pCa 4.0 + calmodulin + W-7	0.110

Fig. 4. The insulin-receptor-cytoskeleton complex

IM-9-cell membrane fractions (with or without ^{125}I -insulin treatment) were extracted with Triton X-100, followed by centrifugation at $110\,000\ g_{av}$ for 1 h. The supernatant fraction was then mixed with anti-(insulin receptor)-conjugated immuno-beads by the procedures described in the Materials and methods section. Autoradiogram (a) and Coomassie Blue staining (b) of cellular materials isolated by anti-insulin-receptor-conjugated immuno-beads. (a) Autoradiogram showing the presence of ^{125}I -insulin (^{125}Ins) binding site (a 130 kDa protein, the α -subunit of insulin receptors) in the anti-insulin-receptor immuno-beads. (b) Coomassie Blue staining of (a), showing materials co-isolated with 130 kDa insulin receptor in the anti-insulin-receptor immuno-beads; note that actin, myosin heavy chain (Myosin HC) and myosin light chain (Myosin LC) were observed in this preparation, designated as the insulin-receptor-cytoskeletal complex. (c) Coomassie Blue staining of cellular materials which were non-specifically bound to a column made with antiserum pre-absorbed with IM-9-cell insulin receptors (anti-insulin-receptor-free serum); note that very little non-specific immunobinding was observed. (No detectable ^{125}I -insulin label was observed in this preparation; results not shown.) (d) Autoradiogram of immunoblot of the insulin-receptor-cytoskeleton complex [prepared by procedures described for (a) and (b) above] incubated with anti-(smooth-muscle myosin light-chain) antibody and then with ^{125}I -Protein A. (e) Autoradiogram of ^{32}P -labelled proteins from the insulin-receptor-cytoskeleton complex. Protein phosphorylation was carried out in the presence of high Ca^{2+} (0.1 mM; pCa 4.0; and calmodulin. The insulin receptor-cytoskeleton complex [prepared by procedures described for (a) and (b) above] was used as a crude MLCK source. The reaction was initiated by the addition of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and incubated for 5 min at 30°C as described in the Materials and methods section. (Note that a major phosphoprotein at 20 kDa, corresponding to the myosin light chain, and a minor phosphoprotein at 95 kDa, possibly β -subunit of the insulin receptors, were observed). (f) Coomassie Blue staining of the anti-(smooth-muscle myosin light-chain) antibody-mediated immunoprecipitation of mouse T-lymphoma myosin light chain (Myosin LC) along with myosin heavy chain (Myosin HC) (IgG_H, heavy chain of immunoglobulin; IgG_L, light chain of immunoglobulin). (g) Coomassie Blue staining of the anti-(smooth-muscle myosin light-chain) antibody-mediated immunoprecipitation of smooth-muscle myosin light chain along with myosin heavy chain. (h) Coomassie Blue staining of the anti-myosin-light-chain-free serum (i.e. pre-absorbed with purified smooth-muscle myosin light chain)-mediated precipitation of smooth-muscle myosin. (Note that only a very little non-specific precipitation was observed.) M_r standards used were: myosin, 200 000; β -galactosidase, 130 000; phosphorylase b, 94 000; actin, 42 000; concanavalin A, 25 000; soya-bean trypsin inhibitor, 17 000.

DISCUSSION

The present study demonstrates that insulin, a monovalent ligand, is capable of inducing its receptors on the surface of IM-9 human lymphoblasts to aggregate into patches and caps. This insulin-induced receptor movement appears to have all the characteristics of well-known multivalent ligand (e.g. lectin and antibody)-induced receptor capping [11]. It has been suggested that receptor patching and capping may be responsible for a number of cellular functions, including insulin-dependent glucose oxidation [32]. In view of the importance of the physiological effects that may be caused by receptor

patching and capping, we decided to investigate further the regulatory mechanism and structural reorganization involved in the redistribution of insulin-bound surface receptors.

Previously, we were unable to detect any insulin-induced change in internal free Ca^{2+} concentration by using IM-9 cells loaded with the fluorescence Ca^{2+} indicator quin-2 [33]; despite other evidence suggesting that a calmodulin-dependent process is involved in the insulin-produced receptor motility [11]. In the present study, a newly developed fluorescent Ca^{2+} indicator (fura-2) has been used to re-examine the effect of insulin on intracellular Ca^{2+} activity. As one of the 'second

generation' Ca^{2+} probes developed by Tsien *et al.* [33], fura-2 displays a much higher (at least 200-fold) sensitivity than quin-2. Consequently, we are able to demonstrate a rise in intracellular Ca^{2+} concentration upon binding of insulin to the IM-9 cells loaded with fura-2 (Fig. 1). Although the maximum Ca^{2+} concentration is low compared with that in muscle, it is a consistently reproducible change. A small local increase in Ca^{2+} concentration along the cytoplasmic surface of the plasma membrane may be sufficient to activate pertinent cellular processes such as actomyosin contraction. The increase in internal Ca^{2+} occurred before the onset of insulin-receptor capping (Figs. 1a and 1b) as would be expected if Ca^{2+} were the second messenger. In smooth muscle, the intracellular free Ca^{2+} concentration reaches a maximum before contraction [34]. Thus important similarities with regard to internal Ca^{2+} -concentration patterns exist between muscle contraction and insulin-induced receptor capping.

The regulatory molecule calmodulin has been implicated in many physiological processes [29]. Graves *et al.* have shown that insulin receptors are closely associated with a calmodulin-binding moiety [24] and that calmodulin may be involved with insulin-induced insulin-receptor phosphorylation [35]. We previously reported that the calmodulin inhibitors W-5, W-7, W-12 and TFP inhibit insulin-receptor capping in a dose-dependent manner [11]. The facts that (1) a number of calmodulin inhibitors, such as W-7 and TFP, inhibit insulin-induced receptor capping and myosin light-chain phosphorylation (Table 1b) and (2) intracellular calmodulin molecules are concentrated directly underneath the insulin-bound capped structures (Figs. 2a and 2b) suggest that calmodulin could be directly involved in insulin-induced capping and myosin light-chain phosphorylation events.

The myosin molecule consists of six polypeptides: one pair of heavy chains (each 200 kDa) and two pairs of light chains of variable molecular mass (15–27 kDa), depending on the source of myosin [3]. These proteins are associated to form two main functional domains. The C-terminal halves of the two heavy chains associate into an α -helical coil which is involved in supramolecular filament formation (rod or tail domain), and each N-terminal half forms a globular domain associated with two light chains [36]. Phosphorylation of the myosin heavy chain has been described in several vertebrate [37] and invertebrate [38] systems, but as yet heavy-chain phosphorylation has no known physiological function. However, phosphorylation of myosin light chain has been shown to stimulate the actin-activated Mg^{2+} -ATPase of myosin, which releases the energy to drive the sliding filament contraction process in non-muscle cells [2,13]. Studies on both smooth-muscle and non-muscle cells indicate that actomyosin-mediated contraction is regulated by a Ca^{2+} /calmodulin-dependent MLCK [39–42]. MLCK has been identified in lymphocytes as a 130 kDa protein in the plasma-membrane-cytoskeleton subcellular fraction [16]. In the present study we have used a specific antibody raised against chicken gizzard MLCK [43] to localize the MLCK in IM-9 cells. Our results indicate that some intracellular MLCK accumulates directly underneath the insulin-induced capped structure (Figs. 2c and 2d). This enzyme, which is closely associated with insulin-receptor-actomyosin complex, catalyses myosin light-chain phosphorylation

in a Ca^{2+} /calmodulin-dependent manner (Table 2). In unstimulated IM-9 cells (in the absence of insulin treatment), MLCK exhibits a relatively low activity (Table 2a). In the presence of insulin, there is a remarkable increase in the MLCK activity (Table 2b). In addition, we have shown that, on treatment with calmodulin-binding drugs such as TFP and W-7, insulin-receptor capping and MLCK activity are concomitantly inhibited (Table 1) [11]. This evidence strongly suggests that Ca^{2+} /calmodulin-dependent MLCK is involved in insulin action.

At present, it is not yet clear what exactly constitutes the transmembrane linkage between insulin receptors and the complex of actomyosin, MLCK and calmodulin. Insulin receptors may become associated with cytoskeletal components directly or via certain membrane-attached proteins (e.g. ankyrin or band-4.1-like-protein proteins), which subsequently bind to fodrin (a spectrin-like actin-binding protein) and/or actin, as described previously in mouse T-lymphoma membranes [44–48].

On the basis of the evidence presented in this study that (1) increases in intracellular Ca^{2+} precede insulin-induced receptor capping, (2) insulin receptors, actomyosin, MLCK and calmodulin are located in the cap structure, (3) inhibitors of calmodulin prevent insulin-induced capping and myosin light-chain phosphorylation, and (4) myosin light-chain phosphorylation correlates with capping, we propose that Ca^{2+} /calmodulin-dependent MLCK-mediated myosin light-chain phosphorylation is critically important in the actomyosin-mediated contractility required for collecting the insulin receptors into caps.

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