Staurosporine inhibits phorbol 12-myristate 13-acetate- and insulin-stimulated translocation of GLUT1 and GLUT4 glucose transporters in rat adipose cells

Haruo NISHIMURA* and Ian A. SIMPSON†

Experimental Diabetes, Metabolism and Nutrition Section, Diabetes Branch, National Institute of Diabetes and Digestive and Kidney Disease, National Institutes of Health, Bethesda, MD 20892, U.S.A.

Staurosporine, a widely used protein kinase C inhibitor, completely inhibited both phorbol 12-myristate 13-acetate (PMA)- and insulin-stimulated glucose transport activity in isolated rat adipocytes. The inhibition was non-competitive and was attributed to a blockade of the PMA- and insulin-induced translocation of both GLUT1 and GLUT4 glucose transporters. The PMA-stimulated glucose transport activity was more sensitive to inhibition by staurosporine than was insulin-stimulated transport activity (PMA, $IC_{50} = 1.1 \pm 0.1 \mu M$; insulin, $IC_{50} = 6.4 \pm 0.7 \mu M$; $P < 0.05$, $n = 3$). At $1 \mu M$ staurosporine the insulin-sensitivity was decreased, i.e. EC_{50} increased from 0.12 nM to 5.4 nM , but the maximum response to insulin and the time course for stimulation were unaffected. At $6 \mu M$ staurosporine the insulin-sensitivity was further decreased, the maximal stimulation was decreased by 25% , and the apparent half-time for stimulation was extended from 2.5 min in control cells to

9.4 min . Staurosporine ($30 \mu M$) was able to block insulin's ability to stimulate glucose transport, whether added before or after insulin, by a mechanism that did not alter the rate of GLUT4 internalization. In intact adipose cells, staurosporine ($30 \mu M$) induced a slight (30%) decrease in the maximal insulin-induced receptor autophosphorylation and a similar decrease in the tyrosine phosphorylation of pp60 and pp160 (insulin-receptor substrate-1: 'IRS-1'), but was without effect on insulin binding to its receptor. Conversely, staurosporine induced a concentration-dependent inhibition of the constitutively tyrosine-phosphorylated (pp120) protein and of an insulin-stimulated protein pp53 in the cytosol. The locus of staurosporine's action appears to be distal from the initial insulin-receptor signalling, at a step that regulates the specific translocation of the glucose transporters to the plasma membranes.

INTRODUCTION

Considerable advances have been made in our understanding of the mechanisms by which hormone-receptor tyrosine kinases, and the insulin-receptor kinase in particular, elicit their stimulatory effects on cellular growth and differentiation. It is now known that these signal-transduction pathways involve a cascade of protein interactions involving insulin-receptor substrate-1 (IRS-1), ras, raf, MEK and mitogen-activated protein (MAP) kinase, although the precise sequences of interactions have yet to be confirmed (for reviews see [1–4]). By contrast, little information is available concerning the mechanism(s) by which the acute insulin effects on lipid and carbohydrate metabolism are mediated. Recently a potential link with the growth and differentiation cascades has been identified in the form of an insulin-sensitive kinase in skeletal muscle that exhibits similar properties to an S6 kinase in oocytes [5,6]. Both enzymes were found to be stimulated by MAP kinase and the muscle protein was found to stimulate a phosphatase that dephosphorylates and activates glycogen synthase.

The involvement of Ca^{2+} /lipid-dependent protein kinase (protein kinase C) in the mediation of acute responses to insulin action continues to be controversial [7,8]. We and others have reported that protein kinase C activity is stimulated by insulin

[9–11], and that activators of protein kinase C, e.g. phorbol 12-myristate 13-acetate (PMA) or vasopressin, can stimulate glucose transport activity [9–16] by inducing a translocation of both GLUT1 and GLUT4 glucose transporters [13,15–18]. In our studies, the GLUT1 translocation is comparable with that elicited by insulin; however, the GLUT4 translocation was only 10% of that induced by insulin [15], but greater GLUT4 responses were observed by Häring et al. [16].

One criterion frequently invoked to link protein kinase C and insulin action has been based on the use of dual-action inhibitors. A diverse collection of inhibitors of protein kinase C, including sphingosine, polymyxin B, H7, sangivamycin and staurosporine [17–21], have been demonstrated to inhibit many of insulin's actions in a variety of cell types. However, in all cases, questions as to the specificity of these reagents in intact cells have cast doubt on the specific involvement of protein kinase C in mediating insulin action. For example, staurosporine, the most potent inhibitor of protein kinase C ($IC_{50} = 2.7 \text{ nM}$) [22], has been shown to inhibit cyclic AMP-dependent protein kinase and pp60 v-src tyrosine protein kinase [23–25]. More recently, studies *in vitro* revealed staurosporine's ability to block both the autophosphorylation and the tyrosine kinase activity of the insulin receptor ($IC_{50} = 68 \text{ nM}$) [26] and a soluble vanadate-stimulated tyrosine kinase in adipose cells [27,28].

Abbreviations used: GLUT, glucose transporter isoform; IRS-1, insulin-receptor substrate-1; MAP kinase, mitogen-activated protein kinase; PAO, phenylarsine oxide; PMA, phorbol 12-myristate 13-acetate.

* Present address: Department of Endocrinology and Metabolism, Shizuoka General Hospital, 4-27-1 Kitaado, Shizuoka 420, Japan.

† To whom reprint requests should be addressed, at: EDMNS/DB/NIDDK, Bldg. 10, Rm 5n102, National Institutes of Health, Bethesda, MD 20892, U.S.A.

In an earlier study by Standaert et al. [19], insulin- and PMA-stimulated glucose transport activity was shown to be inhibited by staurosporine in isolated rat adipose cells. In this study we have confirmed these original observations and have further investigated the locus at which staurosporine elicits its inhibitory response.

EXPERIMENTAL

Animals and cell preparation

Male rats (170–200g; CD strain, Charles River Breeding Laboratories) were used throughout. The rats were anaesthetized with a gas mixture of CO₂/O₂ (7:3) and killed by decapitation. The epididymal fat-pads were removed, minced and digested with collagenase (type 1, Cooper Biochemical) as described previously [29].

Incubation of adipose cells and determination of glucose transport activity

All incubations were carried out at 37 °C in a Krebs–Ringer bicarbonate–Hepes buffer, pH 7.4, containing 10 mM NaHCO₃, 30 mM Hepes, 200 nM adenosine and 5% (w/w) BSA (fraction V; Reheis Chemical Co.). Isolated adipose cells were distributed equally into plastic vials in a final incubation volume of 400 µl containing the indicated reagents. PMA was dissolved in dimethyl sulphoxide, and all cells were exposed to a final concentration of dimethyl sulphoxide of 0.1%. At the end of the incubation period, glucose transport activity was determined by the 3-*O*-methylglucose uptake technique as described previously [29]. For equilibrium exchange studies, adipose cells were equilibrated with the various concentrations of 3-*O*-methylglucose (0–50 mM) for 60 min before measurement of initial velocities [30].

Preparation of subcellular membrane fractions

The adipose cells obtained from 50 to 56 rats were incubated at 37 °C in polypropylene containers, each containing a total volume of 40 ml [(0.8–1.2) × 10⁸ cells/ml]. The cells were preincubated either with or without staurosporine for 30 min, before a 30 min incubation either with no further additions (basal), or with 0.7 µM insulin or 1 µM PMA. 3-*O*-Methylglucose transport activity was assayed in 200 µl samples of cells as described previously [29]. Cells were centrifuged through dinonyl phthalate, resuspended in TES buffer (20 mM Tris, 1 mM EDTA, 255 mM sucrose) containing 0.2 mM Na₃VO₄, 10 mM Na₄P₂O₇ and 10 mM NaF, pH 7.4, 17 °C, and homogenized. Plasma membranes (PM) and intracellular membranes (IM1 and IM2; previously inappropriately referred to as ‘high-density microsomes’ and ‘low-density microsomes’ respectively [31]) were prepared by differential centrifugation as described previously [29,32].

Immunoblotting of glucose transporters

Each membrane fraction was subjected to SDS/PAGE and Western-blot analysis with polyclonal antibodies raised against the C-terminal peptides of GLUT1 (379) and GLUT4 (α-GT4) as described previously [32]. The relative glucose transporter concentrations were revealed by using ¹²⁵I-labelled Protein A and quantified by excising the appropriate band and γ-radiation counting.

Insulin binding

Plasma membranes (20 µg) were incubated with ¹²⁵I-insulin tracer with various concentrations of unlabelled insulin (0–0.7 µM) at 4 °C for 12 h. The membranes were centrifuged for 30 min at 100 000 g, the supernatant was discarded, and the bound insulin was determined by γ-radiation counting. Non-specific binding was determined in the presence of 7 µM unlabelled insulin.

Intact-cell autophosphorylation and tyrosine kinase activity of the insulin receptor

Membrane fractions or whole cell lysates were used for Western blotting with a rabbit polyclonal anti-phosphotyrosine antibody (α-PTY) (purchased from Zymed, San Francisco, CA, U.S.A.). In whole-cell experiments, cells (250 µl) were preincubated with various concentrations of staurosporine (0–30 µM) and then incubated with insulin (0.7 µM). After 1 min or 30 min, cells were washed with TES (4 °C) containing protease and phosphatase inhibitors solubilized in sample buffer containing phosphatase inhibitors, (5% SDS, 50 mM Tris, 2 mM Na₃VO₄, 10 mM NaF, 10 mM Na₄P₂O₇, 0.5 µg/ml pepstatin, 1 µM phenylmethanesulphonyl fluoride, 0.5 µg/ml aprotinin, 1 µg/ml leupeptin, 50 mM dithiothreitol, pH 6.8), and boiled for 3 min; the infranatant was collected as the sample. After determining the protein concentration (BCA assay, Sigma), dithiothreitol (50 mM) was added, and the samples were boiled for an additional 3 min and subjected to SDS/PAGE and Western blotting.

Calculations

Curve fitting was performed with the MLAB program (Civilized Software Inc., Bethesda, MD, U.S.A.). Statistical significance was tested with analysis of variance (ANOVA) following Duncan's multiple range test or paired *t*-test when appropriate, and differences were accepted as significant at the *P* < 0.05 level.

RESULTS

To determine whether the signalling mechanisms by which insulin and PMA stimulate glucose transport activity share a common intermediate, the sensitivities to staurosporine inhibition of transport activity were compared (Figures 1a and 1b). Isolated adipocytes were exposed to the indicated concentrations of staurosporine for 30 min and subsequently incubated for an additional 30 min either with no further addition (basal), or with insulin (0.7 µM) as illustrated in Figure 1(a), or PMA (1 µM) as shown in Figure 1(b). Comparison of Figures 1(a) and 1(b) reveals that staurosporine is able to inhibit completely both PMA- and insulin-stimulated activities. However, the PMA-stimulated transport activity was more sensitive to inhibition by staurosporine (IC₅₀ 1.1 ± 0.1 µM) than insulin-stimulated activity (IC₅₀ 6.4 ± 0.7 µM, *P* < 0.05, *n* = 3), thus providing support for different signalling pathways.

To determine the mechanism by which this inhibition is mediated, insulin-stimulated 3-*O*-methylglucose transport activity was measured by equilibrium exchange (see the Experimental section) in the presence and absence of an intermediate concentration of staurosporine (6 µM). From the Lineweaver–Burk plots shown in Figure 2, it is apparent that the primary action of staurosporine is to decrease the *V*_{max} for transport activity by 33% (0.15 ± 0.02, cf. 0.1 ± 0.01 pmol/min per cell), while having no effect on *K*_m of the transporters for 3-*O*-methylglucose (control cells, 4.6 ± 0.2 mM; staurosporine-treated cells, 4.5 ± 0.3 mM).

Figure 1 Inhibition of basal, insulin- and PMA-stimulated glucose transport activity: staurosporine concentration-dependence

Isolated fat-cells were exposed to various concentration of staurosporine for 30 min, incubated with no further additions (BAS), or with insulin (INS; 0.7 μM) or PMA (1 μM) for 30 min, and subsequently 3-O-methylglucose transport activity was determined as described in the Experimental section. Values are means ± S.E.M. for triplicate determinations in a representative study from 3 independent experiments.

Figure 2 Effects of staurosporine on the kinetics of 3-O-methylglucose transport activity in rat adipocytes

Adipose cells were incubated for 60 min with insulin (0.07 μM) with or without staurosporine (6 μM) in the presence of the indicated concentration of 3-O-methylglucose. Transport was then initiated by addition of [¹⁴C]3-O-methylglucose. Lineweaver-Burk plots of control (CONT; ●) and staurosporine (ST; ○)-treated cells are illustrated.

Insulin and PMA are known to stimulate glucose transport activity ($V_{\max}$) in rat adipose cells by inducing a translocation of GLUT1 and GLUT4 glucose transporters from an intracellular site to the plasma membrane. However, alterations in transporter accessibility, as induced by isoprenaline and adenosine following translocation, also result in changes in $V_{\max}$ [30,31]. It was therefore important to determine the effects of staurosporine on these insulin- and PMA-mediated translocation processes. Cells were preincubated with staurosporine and subsequently exposed to insulin (0.7 μM) or PMA (1 μM). The cells were washed and fractionated as described in the Experimental section, and the presence of GLUT1 and GLUT4 in the corresponding plasma membranes and intracellular membranes was determined by

Figure 3 Effects of staurosporine on subcellular distribution of glucose transporters: Western blotting of GLUT1 and GLUT4

Isolated fat-cells were incubated in the absence or presence of 30 μM staurosporine for 30 min, and then cells were incubated with no further additions (BAS), 0.7 μM insulin (INS) or 1 μM PMA for 30 min. Cells were fractionated, and plasma membranes and intracellular membranes were subjected to SDS/PAGE. Western blots were performed using anti-GLUT1 and -GLUT4 antibodies and ¹²⁵I-protein A. Data illustrate a typical experiment.

Figure 4 Effects of staurosporine on the sensitivity of glucose transport stimulation to insulin

Cells were exposed to the various concentrations of staurosporine for 30 min and subsequently incubated with the indicated insulin concentration for an additional 30 min. 3-O-Methylglucose transport activity was determined as described in the Experimental section. The result shown is representative of 3 separate experiments.

Western blotting using specific C-terminal antibodies and ¹²⁵I-Protein A. The data presented in Figure 3 clearly demonstrate that staurosporine blocks the translocation of both GLUT1 and GLUT4, while having no effect on the basal transporter distribution.

To determine the locus of staurosporine's action, its effects on insulin sensitivity were investigated. Adipose cells were exposed to various concentrations of staurosporine for 30 min and subsequently stimulated with insulin for 30 min. As seen in Figure 4, at the lowest concentration of staurosporine (1 μM), it appears that only the sensitivity to insulin is affected and the maximum transport response remains unaffected. At higher concentrations (6 μM), both parameters are altered, and at 30 μM, as seen in Figure 1, all response to insulin is blocked.

The observations presented in Figures 1–4 suggest that staurosporine inhibits the insulin-signalling process rather than glucose transport activity directly. To verify this hypothesis, the tyrosine-phosphorylation state of the β-subunit of the insulin receptor and the prominent tyrosine-phosphorylated substrates were monitored as a function of staurosporine concentration (Figure 5a). Adipose cells (1 × 10⁵/200 μl) were preincubated with the

Figure 5 Effects of staurosporine on autophosphorylation and tyrosine kinase activity of insulin receptors

Cells were preincubated with various concentrations of staurosporine (ST) for 10 min and then incubated with insulin (INS; 0.7 μM) for either 1 min or 30 min. Whole-cell extracts were subjected to SDS/PAGE and Western blotting. Phosphotyrosine-containing proteins were detected with anti-phosphotyrosine antibody and 125 I-Protein A. The level of phosphorylation of pp95, the β -subunit of the insulin receptor, and pp160 (IRS-1), illustrated in (a), were quantified by excising the respective bands and counting γ -radiation. In (b) the staurosporine concentration-dependence for inhibition of the phosphorylation of pp95 and pp160 is compared with its effects on PMA- and insulin-stimulated glucose transport activity. The result shown is representative of 3 separate experiments.

indicated staurosporine concentrations, followed by a supra-maximal concentration of insulin (0.7 μM) for either 1 or 30 min. The cells were then solubilized as described in the Experimental section, and the phosphotyrosine-containing proteins pp160, pp120, pp95, pp60 and pp53 were detected by Western blotting with an anti-phosphotyrosine antibody and 125 I-Protein A as depicted in Figure 5(a). After a 1 min incubation, insulin enhanced the phosphorylation of both pp160 (corresponding to IRS-1) and pp60 approx. 3-fold, and the phosphorylation of both was only minimally decreased by the presence of staurosporine. Insulin also enhanced the tyrosine phosphorylation of the β -subunit of the insulin receptor, pp95, approx. 2.7-fold, and staurosporine decreased this level by 30% at both time points. Overall, there was an approx. 10% decrease in the level of phosphotyrosine phosphorylation as a result of the 30 min insulin incubation relative to the 1 min incubation, suggesting that transient changes in phosphorylation are not responsible for the observed alterations in transport activity.

The effects of staurosporine on glucose transport activity stimulated by PMA or insulin are compared with the changes in the phosphorylation state of the receptor and primary substrates in Figure 5(b). Clearly the effects on transport activity are far more profound than on the receptor or IRS-1 tyrosine phosphorylation, suggesting that staurosporine's action is distal to the initial receptor activation. After a 1 min exposure to insulin, and to a lesser extent after 30 min (Figure 5a), there appears to be an

Figure 6 Effect of staurosporine on the time course for insulin stimulation

Adipose cells were incubated with the various concentrations of staurosporine for 30 min. Insulin (0.7 μM) was added at zero time and 3-O-methylglucose transport activity was determined at the indicated times. The result shown is representative of 3 separate experiments.

increase in the tyrosine phosphorylation of a protein of approx. 53 kDa that is inhibited by staurosporine. This protein may well correspond to the cytoplasmic vanadate-stimulated tyrosine kinase described by Shisheva and Shechter [27,28], which is also known to be inhibited by staurosporine. Another intriguing observation is the concentration-dependent inhibition of the constitutive tyrosine phosphorylation of pp120 protein, a protein whose phosphorylation state is unaffected by insulin. It should be noted that insulin binding to isolated plasma membranes prepared from cells exposed to 30 μM staurosporine was unaffected, with bound insulin/total insulin of $14.0 \pm 0.4\%$ for membranes from control cells and $14.5 \pm 0.3\%$ for staurosporine-treated cells ($n = 3$). The affinity for insulin was also unaffected (results not shown).

Recent studies on the translocation mechanism(s) have suggested that GLUT4 transporters, once recruited to the plasma membrane, continuously recycle between their intracellular location(s) and the plasma membrane [33–36]. Furthermore, it has been proposed that insulin accelerates the exocytic movement of GLUT4, resulting in the steady-state redistribution of the transporters [33–36]. It was therefore important to determine the effects of staurosporine on the time courses for the stimulation and reversal of insulin action. Cells were preincubated with various concentrations of staurosporine, and insulin (0.7 μM) was added at zero time; 3-O-methylglucose transport activity was measured at the indicated times (Figure 6). The half-time for stimulation in control cells was 2.5 ± 0.2 min, which was unaffected by 1 μM staurosporine, a concentration which had altered the sensitivity of the transport response to insulin (Figure 4). At higher concentrations (6 μM), the maximum response to insulin was decreased and the half-time considerably extended to 9.3 ± 0.7 min. The data presented in Figure 6 could potentially result from an accelerated loss of transporters from the plasma membrane. To investigate this possibility, the time course for the reversal of insulin-stimulated glucose transport activity, induced by degrading the insulin with collagenase, was compared with the action of staurosporine. Isolated cells were incubated with insulin (0.7 nM in the collagenase experiment; 0.7 μM in the staurosporine experiment) for 30 min. Collagenase (1 mg/ml) or staurosporine (30 μM) was added at zero time and 3-O-methylglucose transport was measured at the indicated times. Figure 7 shows that the time courses for the reversal are essentially

Figure 7 Reversal of insulin-stimulated glucose transport activity: comparison of staurosporine and collagenase effects

Adipose cells were incubated for 30 min with insulin (INS) (0.7 nM in collagenase experiment and 0.7 μ M in staurosporine experiment). Staurosporine (ST; 30 μ M) or collagenase (COLL; 1 mg/ml) was then added, and 3-O-methylglucose transport activity was determined at the indicated times. The result shown is representative of 3 separate experiments. Basal transport activity was determined at the indicated times.

Figure 8 Time course for the reversal of insulin-stimulated translocation of GLUT1 and GLUT4 by staurosporine: Western-blot analysis of plasma membranes and intracellular membranes

Adipose cells were exposed to insulin (INS) and subsequently to staurosporine (ST) as described for Figure 7. The cells were fractionated and the isolated membranes analysed for GLUT1 and GLUT4 content by Western blotting as described in the Experimental section. The result shown is representative of 3 separate experiments.

identical, with apparent half-times of 12 min. In parallel experiments, adipose cells were fractionated at various times and the isolated subcellular fractions blotted for GLUT1 and GLUT4. The reversal of transport activity is accompanied by a comparable loss of both transporter isoforms from the plasma membranes and a concomitant re-appearance in the intracellular membrane fractions (Figure 8). Thus the locus of staurosporine action would appear to be distal to the insulin/insulin-receptor interaction, but between the receptor and the initial translocation events that lead to externalization of the transporters.

DISCUSSION

Since the initial description of staurosporine [22], its specificity has been drawn into question. Initially it was described as the most potent inhibitor of protein kinase C, and several publications have used this property as a criterion to implicate the involvement of protein kinase C in certain biological actions. This has been particularly true in studies of insulin action where

staurosporine and other protein kinase C inhibitors such as H-7, sphingosine and polymyxin B have been shown to inhibit insulin action in a wide variety of cells [18–21], thus implying a role for protein kinase C in the mediation of insulin signalling. Further support for this concept was provided by the direct demonstration of insulin-stimulated protein kinase C activity [9–11] and the translocation of specific protein kinase C isoforms to the plasma membranes in response to insulin (as reviewed in [7]). However, more recent studies have indicated that the specificity of all of these inhibitors, and staurosporine in particular, is not as confined as originally thought. Staurosporine has since been found to inhibit cyclic AMP-dependent protein kinase [23,24], p60src, a tyrosine kinase [25], and even the insulin receptor itself [26], raising significant doubts as to its locus of action. In this study we have confined our study to the mechanism by which staurosporine inhibits insulin- and PMA-stimulated glucose transport activity. We are able to confirm the initial observations of Standaert et al. [19] that staurosporine completely inhibits both insulin- and PMA-stimulated glucose transport activities in rat adipose cells, but with differential sensitivities, i.e. the IC_{50} for PMA-stimulated transport activity was 1 μ M, compared with 6 μ M for the inhibition of insulin-stimulated activity. Although such observations suggest that insulin and PMA elicit their stimulatory effects on glucose transport activity by different mechanisms, they do not exclude a role for protein kinase C in insulin action. It is conceivable that other scenarios might explain the present observations. For example: (1) PMA and insulin activate two distinct protein kinase C isoforms, α and β_2 , respectively [14], which may exhibit differential sensitivities to staurosporine; (2) the sensitivity of protein kinase C α to staurosporine inhibition is greater than the subsequent event that mediates both insulin's and PMA's action on the translocation of GLUT1 and GLUT4; (3) a third, and most attractive, possibility is that staurosporine is inhibiting two distinct signalling systems, which would account for the different degrees of GLUT1 and GLUT4 translocation [15–17].

As indicated above, the specificity of staurosporine has been shown to be less than absolute, and several other protein kinases, including the insulin receptor kinase, are susceptible to inhibition. Of particular interest is the rat adipocyte tyrosine kinase characterized by Schechter and colleagues [27,28] as a cytosolic 53 kDa protein which appears to undergo autophosphorylation. This protein is believed to mediate vanadate's stimulation of glucose oxidation and glucose incorporation into lipid, and is extremely sensitive to inhibition by staurosporine. However, vanadate activation of glucose transport activity was apparently not staurosporine-sensitive, and it therefore was concluded that this kinase was not involved in the activation of transport. In this study we have identified a cytosolic protein of approx. 53 kDa that is tyrosine-phosphorylated in response to insulin and is inhibited, in a concentration-dependent manner, by staurosporine (Figure 5a). It should be noted that the concentration of staurosporine required to inhibit intact cell activities is considerably higher than that observed to inhibit the cytosolic tyrosine kinase [26]. In this study, and in the studies of Standaert et al. [19], still higher concentrations of staurosporine were found necessary to inhibit insulin-stimulated glucose transport activity. The concentration-dependence for the inhibition of transport activity is clearly similar to that seen for the decrease in tyrosine phosphorylation of the 53 kDa protein, and the potential involvement in transporter translocation remains to be determined. Interestingly, staurosporine also causes a dose-dependent inhibition of the tyrosine phosphorylation of the cytosolic protein pp120, the function of which remains to be determined. This protein is distinct from a similar molecular-mass protein found

in rat hepatocytes, also referred to as pp120, which is phosphorylated in response to insulin (S. Taylor, personal communication).

The effects of staurosporine on the tyrosine phosphorylation of the insulin receptor and IRS-1 (pp160), seen in Figure 5(a), are clearly distinct from the effects on pp53 and pp120. The tyrosine-phosphorylation state of both the insulin receptor and one of its primary substrates, IRS-1, is only marginally decreased by staurosporine under circumstances where transport is completely inhibited, suggesting that in the intact cell the inhibition of the insulin receptor kinase activity is not responsible for the loss of transport stimulation. The spare-receptor hypothesis, originally formulated by Kono and Barham [37], suggests that only 5–10% of the insulin receptors need to be activated to elicit a full stimulation of transport activity, whereas up to 80% of the receptor tyrosine phosphorylation and activity, based on IRS-1 phosphorylation, remains when transport is completely inhibited by staurosporine, suggesting that the locus of staurosporine inhibition is distal to the insulin receptor and IRS-1.

Kinetically, we were able to demonstrate that staurosporine decreased the V_{max} for insulin-stimulated glucose transport activity, but was without effect on the affinity (K_m) of the transporter for 3-*O*-methylglucose. Furthermore, staurosporine had no effect on basal activity, suggesting an inhibition of the translocation process and not a direct interaction with the transporters (Figure 2). This was confirmed by Western blotting of membranes prepared from cells that had been either (1) exposed to staurosporine before addition of either insulin or PMA, where no translocation of either GLUT1 or GLUT4 was observed (Figure 3), or (2) exposed to insulin and then staurosporine, where the decline in the GLUT4 concentration in plasma membrane correlated well with the decrease in transport activity (Figure 7b). Several recent studies have demonstrated that, upon insulin stimulation, the transporters recruited to the plasma membrane continue to recycle between the plasma membrane and their intracellular location [33–36]. Consequently, the observed effects of staurosporine could result from either accelerating the internalization of the transporters or blocking their recruitment. From Figures 6, 7 and 8 it would appear that staurosporine elicits the latter response. At low concentrations, only the time course (half-time) for translocation appears to be slowed. However, at higher concentrations, the translocation half-time is dramatically increased (9.5 min, cf. 2.5 min in control cells) and the maximal response is also diminished. This effect is independent of the insulin concentration and, as such, represents the first demonstration of a slowing of the translocation response other than by lowering the temperature [38,39].

Many of the effects of staurosporine are reminiscent of the effects of phenylarsine oxide (PAO) on glucose transport activity in 3T3-L1 adipocytes [40–43]. Both agents inhibit insulin-stimulated glucose transport activity, but have little or no effect on basal activity. Neither compound significantly alters the autophosphorylation *in vivo* of the insulin receptor or its activity, whether measured *in vitro* by Frost et al. [41] or *in vivo*, in the present study, by monitoring the phosphorylation of the principal substrate IRS-1. PAO has also been shown to reverse the action of insulin, as seen for staurosporine, by inducing an internalization of GLUT4. However, the action of the two agents are distinct. PAO has little effect on GLUT1 translocation; by contrast, staurosporine blocks the translocation of both GLUT1 and GLUT4 transporters to the plasma membrane in response to insulin or PMA. It also promoted the internalization of both transporters when insulin-treated cells were subsequently exposed to staurosporine (Figures 2 and 8 respectively). Given that PMA, in comparison with insulin, preferentially promotes the trans-

location of GLUT1 and is more sensitive to inhibition by staurosporine, it could be suggested that GLUT1 translocation is more sensitive to staurosporine inhibition and the signalling mechanism is distinct from that for GLUT4 [42]. These distinctions may equally reflect the difference in cell type, as the ratio of plasma membrane GLUT1 to GLUT4 in 3T3-L1 is approx. 1:1, compared with 1:20 in insulin-stimulated rat adipose cells. We have been unable to carry out comparable PAO studies in rat adipose cells as, in our hands, the cells became extremely leaky after exposure to PAO. However, another inhibitor, ML-9, a blocker of myosin-light chain kinase [44], has recently been shown to inhibit insulin-stimulated glucose transport activity in 3T3 L1 cells [45]. Similarly to the action of staurosporine described here, ML-9 exerts its inhibitory action on GLUT4 translocation at a site distal to the insulin receptor and immediate substrates. ML-9 also blocked the activation of both S6 kinase and MAP kinase. However, the involvement of MAP kinase in the translocation process has been questioned by Robinson et al. [46], suggesting a site of ML-9 action before a bifurcation in the insulin-signalling pathways. The ability of staurosporine, PAO and ML-9 to inhibit insulin's ability to stimulate translocation of GLUT4 at seemingly different sites suggests that, as with insulin's effects on growth and differentiation, the translocation process requires the activation of a cascade of kinases, the participants of which may well include protein kinase C, but otherwise have yet to be described. The combined use of 'specific' inhibitors to block the individual steps will clearly be invaluable in the dissection of the overall process.

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