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Effect of phospholipase treatment on insulin receptor signal transduction

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Summary. To study the role of membrane lipids in signal transduction by the insulin receptor, we have studied the effect of phospholipase C *(Clostridium perfringens)* and a phosphatidylinositol-specific phospholipase *(Staphylococcus aureus)* on insulin binding, a function of the α-subunit, and tyrosine kinase activity, a function of the β-subunit in IM-9 lymphocytes and NIH 3T3 fibroblasts transfected with the human insulin receptor. Treatment of the cells with phospholipase C at concentrations up to 3.4 U/ml did not affect specific insulin binding, but reduced insulin-stimulated receptor phosphorylation by 50%. This effect of phospholipase C was observed within 10 min of treatment and occurred with no change in the basal level of phosphorylation. Pre-treatment of cells with insulin for 5 min prior to enzyme addition prevented any change in kinase activity. Insulin-stimulated phosphorylation of pp185, the presumed endogenous substrate for the insulin receptor kinase, was also reduced following phospholipase C treatment, with an almost complete loss of insulin stimulation after exposure of cells to enzyme at concentrations as low as 0.6 U/ml. In contrast to these effects of phospholipase C on intact cells, receptor autophosphorylation was not affected in insulin receptors purified on wheat germ agglutinin-agarose from phospholipase C treated cells. Likewise, the phospholipase C effect was reduced by the addition of phosphatidylcholine, but not by the addition of the protease inhibitors, aprotinin and phenylmethylsulfonyl fluoride, to the incubation indicating its dependence on phospholipid hydrolysis. Treatment of cells with the phosphatidylinositol-specific phospholipase C did not affect any of the parameters studied. These data suggest that the phospholipid environment in the plasma membrane is an important modulator of transmembrane signalling within the insulin receptor heterotetramer and at the level of substrate phosphorylation.

Key words: Membrane lipids, insulin receptors, insulin action, tyrosine phosphorylation, pp185.

Lipids play an important role in the structure and function of plasma membrane [1]. Membrane function is influenced by lipid composition, fluidity of the lipids in the membrane, and lipid-protein interactions [2, 3]. Cell membrane fluidity is known to exert marked effects on the functional properties of many membrane proteins, including the insulin receptor [4–10]. The insulin receptor is an intrinsic membrane glycoprotein composed of two α-subunits (M_r = 135,000) and two β-subunits (M_r = 95,000) joined by disulphide bonds to produce a tetrameric structure [11–14]. Insulin binding to the α-subunit activates the tyrosine kinase in the β-subunit resulting in autophosphorylation of the β-subunit and stimulation of the phosphotransferase activity of the receptor toward tyrosine residues of other proteins [15–17]. Insulin receptor autophosphorylation appears to be a requisite step for the transmission of the insulin signal to metabolic pathways within the cell [18–20], however, the mechanism of the transduction of the signal from α-subunit to β-subunits remains unknown.

Several types of studies have suggested a potential role for membrane lipids in insulin receptor signal transduction [21, 22]. Although the β-subunit of the insulin receptor is a transmembrane protein, evidence has been presented to suggest that both subunits of the receptor are myristoylated, providing a possible interaction of the receptor with membrane lipids [23, 24]. Increasing concentrations of saturated fatty acids in membranes of a variety of cells results in decreased insulin binding via changes in receptor affinity or number [7–10]. Treatment of isolated adipocytes with phospholipase C has also been shown to abolish insulin-stimulated glucose oxidation and anti-lipolysis, although the exact site of action of the enzyme in this context is unknown [25]. Interestingly, insulin treatment of cells appears to result in activation of one or more phospholipases, including a spe-

cific phospholipase C which may generate an inositol glycan derivative capable of mimicking several of the actions of insulin [26–28].

In the present study, we have examined the effects of two different phospholipases on insulin action in NIH 3T3 HIR 3.5 fibroblasts and IM-9 lymphocytes. One is a phospholipase C with a broad specificity isolated from *Clostridium perfringens;* it's major activity is as a phosphatidylcholine phosphohydrolase [29, 30]. The other is a phospholipase C from *Staphylococcus aureus* which is specific for glycosyl-phosphatidylinositol-anchored proteins [31]. We find that treatment of cells with the former, but not the latter, alters signal transduction at the level of tyrosine phosphorylation of the receptor and its primary substrate pp185 (the 185 kilodalton substrate of the insulin receptor kinase).

Materials and methods

Materials

$Na^{125}I$-[Tyr^{A14}]monoiodoinsulin (2000 Ci/mmol) was purchased from Amersham (Boston, Mass., USA); ^{32}P-orthophosphate and γ-^{32}P[ATP] (3000 Ci/mmol) were purchased from New England Nuclear (Boston, Mass., USA); *Clostridium perfringens* phospholipase C (Type XIV) was obtained from Sigma Chemical Company (St. Louis, Mo., USA); phosphatidylinositol-specific phospholipase C was kindly provided by Dr. A. Saltiel (New York, NY, USA). Agarose bound wheat germ agglutinin (WGA-agarose) was obtained from Vector Laboratories (La Jolla, Calif., USA). Tissue culture plasticware was from NUNC (Copenhagen, Denmark). Fetal calf serum, Dulbecco's Modified Eagle's medium (DMEM) and RPMI-1640 were supplied from Gibco (Grand Island, NY, USA). All other chemicals were of analytical grade and obtained from standard suppliers.

Cell culture

All experiments were performed with cultured NIH 3T3 cells transfected with human insulin receptor cDNA (kindly provided by J. Whittaker et al. [32] referred to as NIH 3T3 HIR 3.5 and IM-9 lymphoblastoid cells [33]. The IM-9 cells were grown in suspension in RPMI-1640 with 10 % fetal calf serum; the 3T3 cells were grown in DMEM supplemented with 10 % fetal calf serum. Both cells types were propagated in a humidified atmosphere at 37 °C with 5 % CO_2. The 3T3 cells were subcultured after reaching confluence by 1:5 dilution of cell released from plates by trypsin, whereas the IM-9 cells were subcultured by simple 1:5 dilution.

Insulin binding

Fibroblasts were plated in 10 cm culture dishes. At confluency, the cells were washed twice with 5 ml of binding buffer (100 mmol/l Hepes, 120 mmol/l NaCl, 5 mmol/l KCl, 1.2 mmol/l $MgSO_4$, 8 mmol/l glucose and 1 % bovine serum albumin), pH 8, and incubated with A14-labelled [^{125}I]insulin and various concentrations of unlabelled insulin. After 3 h at 15 °C, the cells were washed three times with cold phosphate buffer, pH 7.4, and solubilized with 0.1 % sodium dodecyl sulphate (SDS), and the radioactivity in the samples was counted. Non-specific binding was determined in parallel samples containing 10 µg/ml of unlabelled insulin. Specific insulin binding (total minus non-specific) was expressed as percent bound per milligram protein [34]. The procedure was essentially the same for IM-9 lymphocytes, except for slight modifications since these cells were in suspension. For IM-9 cells the results were expressed as a percent bound per 1×10^7 cells [33].

Insulin receptor phosphorylation

The NIH 3T3 HIR 3.5 cells were plated onto 10 cm dishes and allowed to reach confluence. The cells were placed in serum-free DMEM with 0.1 % bovine serum albumin for 12 h followed by phosphate-free DMEM containing ^{32}P-orthophosphate (0.1 mCi/ml) for 2 h. The labelled cells were treated with different concentrations of phospholipase C and then stimulated with insulin (1 µg/ml) for 10 min. The reaction was stopped instantaneously by the addition of liquid nitrogen, and the cells allowed to thaw into chilled solubilizing buffer containing 50 mmol/l Hepes, 1 % Triton X-100, 5 mmol/l ethylenediaminetetraacetic acid (EDTA), 100 mmol/l sodium fluoride, 10 mmol/l sodium pyrophosphate, 2 mmol/l sodium orthovanadate, aprotinin (1,000 kallikrein units/ml), and 2 mmol/l phenylmethylsulphonyl fluoride (PMSF), pH 7.4. The cell lysate was centrifuged at 200,000 *g* for 60 min, and the supernatant was incubated with rabbit polyclonal anti-phosphotyrosine antibodies at 4 °C for 10 h. The immune complexes were precipitated by the addition of Protein A (Pansorbin, Calbiochem) as previously described [15] and prepared for SDS-polyacrylamide gel electrophoresis under reducing conditions (100 mmol/l dithiothreitol) according to the method of Laemmli [35]. Autoradiography of the dried gels was performed, and the appropriate areas of the radiographs quantitated by scanning densitometry. The relative level of phosphorylation of the insulin receptor β-subunit and pp185 were expressed as percent of control stimulation of each by insulin alone. In some experiments varying concentrations of phospholipase C were added to some plates of cells for 40 min at 37 °C prior to insulin stimulation. The procedure was essentially the same for IM-9 lymphocytes, except that the cells were in suspension.

Lectin purification of the insulin receptor

The insulin receptor was partially purified from IM-9 lymphocytes or NIH 3T3 cells by wheat germ agglutinin affinity chromatography. Confluent cells were solubilized at 22 °C with 3 ml of 50 mmol/l Hepes, pH 7.4, containing 1 % Triton X-100, 0.1 mg/ml aprotinin and 2 mmol/l PMSF. Following centrifugation to remove the insoluble material, the cell extract was applied to a 1 ml WGA-agarose column, and the insulin receptor was eluted with 1–2 ml of 0.3 mol/l N-acetylglucosamine in 50 mmol/l Hepes, pH 7.4, 0.1 % Triton X-100. Insulin binding was performed on the partially purified receptor preparations at 4 °C for 15 h using the polyethylene glycol precipitation method as previously described [36].

Insulin receptor autophosphorylation

Autophosphorylation of the insulin receptor was studied using partially purified receptor preparations adjusted to give similar insulin receptor concentrations. Insulin binding was performed at 4 °C for 15 h. The sample was then brought to 20 °C, and phosphorylation initiated by adding $MnCl_2$ (3 mmol/l final concentration), 25 µmol/l cold ATP, and 5 µCi [$\gamma^{32}P$]ATP for 10 min. The reaction was stopped by the addition of Laemmli sample buffer and boiling for 3 min. The proteins were separated on 7.5 % polyacrylamide gel electrophoresis according to Laemmli, and the gel was analysed by autoradiography. ^{32}P-incorporation into the β-subunit of the insulin receptor was quantitated by scanning densitometry of the films.

Results

Effect of phospholipase C on insulin receptor and substrate phosphorylation

NIH 3T3 HIR 3.5 fibroblasts were labelled with ^{32}P-orthophosphate then incubated with phospholipase C from *C. perfrigens* for 40 min at 37 °C prior to insulin stimulation. Phosphotyrosine-containing proteins were isolated from solubilized cells using anti-phosphotyrosine antibodies and analysed by SDS electrophoresis. Insulin (1 µg/ml) stimulated the phosphorylation of the 95 kilodalton β-subunit of its own receptor (Fig. 1, lanes 1 and 2). The phosphorylation of the insulin receptor was reduced in a dose-dependent manner when the cells were treated with phospholipase C prior to insulin stimulation (Fig. 1, lanes 3–6). Based on an average of four experiments there was a 30 % reduction in β-subunit after treatment with phospholipase C at a concentration of 0.6 U/ml and a 50 % reduction at a concentration of 3.4 U/ml (Fig. 2). The basal level of receptor phosphorylation, as detected by the anti-receptor antibodies, was not affected by the treatment with phospholipase C (data not shown).

Insulin-stimulated phosphorylation of the presumed endogenous substrate for the insulin receptor kinase pp185 was also reduced by phospholipase C treatment (faint band in Fig. 1). This phosphorylation was more sensitive to phospholipase C treatment with a 60–70 % reduction of pp185 phosphorylation at a phospholipase C concentration of 0.6 U/ml (Fig. 2). Some variability was observed in the control level of insulin stimulation of pp185, probably reflecting the sensitivity of pp185 to phosphotyrosine phosphatases and rapid dephosphorylation or changes in expression in the cell cycle. Treatment of cells with the phosphatidylinositol-specific phospholipase C did not affect the phosphorylation state of insulin receptor or pp185 (data not shown).

The time-course of the phospholipase C effect on receptor autophosphorylation is shown in Figure 3 using 2.3 U/ml of enzyme. The decrease in insulin-stimulated

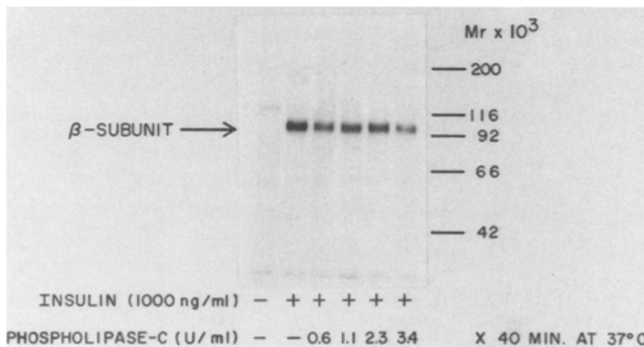

Fig. 1. Effect of phospholipase C treatment on insulin-stimulated receptor phosphorylation in NIH 3T3 HIR 3.5 fibroblasts. Confluent cells were labelled with [^{32}P]orthophosphate as described in Materials and methods, then exposed to phospholipase C for 40 min and finally stimulated with insulin for 10 min. The labelled cells were solubilized, and insulin receptor was immunoprecipitated by anti-phosphotyrosine antibodies. The immunoprecipitates were analysed by SDS-PAGE and autoradiography pp 185 was better visualized in longer exposures

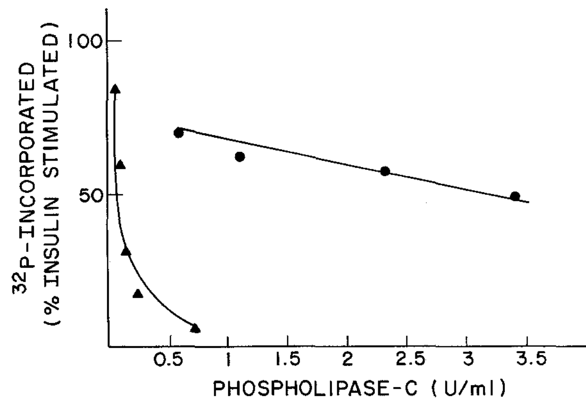

Fig. 2. Effect of phospholipase C on insulin receptor (●) and pp185 (△) phosphorylation. Cells were labelled and phosphoproteins isolated as described in Figure 1. The autoradiograms were then quantitated by scanning densitometry. The amount of phosphate incorporated into the 95 kilodalton (kDa) and 185 kDa bands, expressed as the percent of the insulin-stimulated receptor phosphorylation, is plotted as a function of increasing concentration of phospholipase C. ●—● β-subunit; ▲—▲ pp185

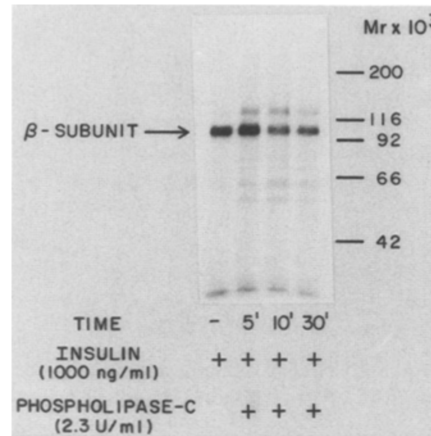

Fig. 3. Time-course of phospholipase C treated cells on insulin-stimulated phosphorylation of insulin receptor in NIH 3T3 HIR 3.5 fibroblasts. Cells were labelled for 2 h with [^{32}P]orthophosphate, then treated with phospholipase C (2.3 U/ml) for 40 min and stimulated with insulin (1 µg/ml) for 10 min. The reaction was stopped by aspirating the medium and freezing the cells with liquid nitrogen. The frozen cells were allowed to thaw into 1 ml of solubilization buffer containing 1 % Triton X-100 and phosphatase inhibitors as described in Materials and methods. The crude extract was immunoprecipitated with anti-phosphotyrosine antibodies, reduced with DTT, and separated by SDS-PAGE. An autoradiogram was prepared with an intensifying screen of the fixed and dried gel and is shown

phosphorylation was seen within 10 min of incubation and did not progress with further incubation to 30 min. No new phosphoproteins or degradation fragments of the β-subunit appeared following phospholipase C treatment.

Effect of phospholipase C on insulin binding and IM-9 lymphocytes

Insulin binding was assessed at 15 °C for 3 h under steady-state conditions. In control NIH 3T3 HIR 3.5 fibroblasts, specific insulin binding was 46.8 %/mg protein with half-maximal inhibition of binding being observed at an unla-

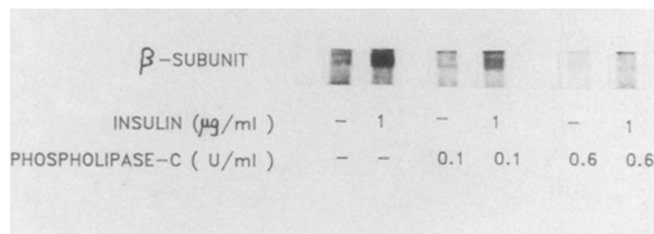

Fig. 4. Effect of phospholipase C treatment on insulin-stimulated phosphorylation of insulin receptor in IM-9 lymphocytes. 1×10^8 cells were labelled with [^{32}P]orthophosphate in phosphate-free medium, then exposed to the phospholipase C for 40 min at 37°C, and finally stimulated with insulin (10^7 mol/l) for 10 min. The cells were centrifuged and the medium aspirated. The cells were immediately solubilized by vortexing in 1 ml of ice-cold solubilizing buffer containing 1% Triton X-100 and phosphatase inhibitors. The crude extracts were immunoprecipitated by anti-phosphotyrosine antibodies and separated by SDS-PAGE. An autoradiogram is shown

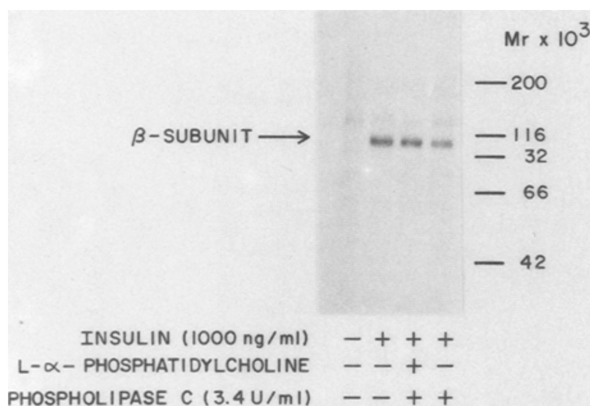

Fig. 5. Effect of L-α-phosphatidylcholine on phospholipase C activity. After labelling with [^{32}P]orthophosphate, the cells were exposed to phospholipase C either in the presence or in the absence of L-α-phosphatidylcholine for 40 min at 37°C. After insulin stimulation the crude extract was immunoprecipitated with anti-phosphotyrosine antibodies. The results of the autoradiography are shown

belled insulin concentration of 12 ng/ml (~2 nmol/l). Treatment of cells with several concentrations of phospholipase C from 0.6 to 3.4 U/ml did not affect either total insulin binding or the affinity of the insulin receptor based on complete competition curves and Scatchard analysis (data not shown). Treatment of IM-9 lymphocytes with phospholipase C also had little or no effect on insulin binding (data not shown). The effect of phospholipase on insulin receptor autophosphorylation, however, was more sensitive in IM-9 cells than with the NIH 3T3 HIR 3.5 cells (Fig. 4). Insulin-stimulated (1 μg/ml) phosphorylation was decreased by approximately 80% with 0.6 U/ml phospholipase C.

The effects of phospholipase C depend on its action on membrane lipids

The effect of phospholipase C is due to its lipolytic action rather than any protease effect. Thus, the inhibitory effect of phospholipase C on receptor autophosphorylation could be abolished by co-incubation of the phospholipase C with L-α-phosphatidylcholine (Fig. 5). In contrast, protease inhibitors, such as aprotinin and PMSF, did not abolish the phospholipase C effect (data not shown). The effect of phospholipase C on receptor phosphorylation was also specific. When total extracts from ^{32}P-labelled cells were analysed there was no major difference in overall phosphoprotein profile between untreated and treated cells (Fig. 6 A), despite the 30–40% reduction of phosphorylation of immunoprecipitated insulin receptor by anti-phosphotyrosine antibodies (Fig. 6 B).

To further validate that the effect of phospholipase C was via its effect on membrane phospholipids, insulin receptors from phospholipase C treated cells were partially purified on WGA-agarose columns and assayed for insulin binding and for insulin-stimulated receptor autophosphorylation in vitro. In this case, no differences were observed between receptors from treated and untreated cells. Moreover, if phospholipase C was added to the cells

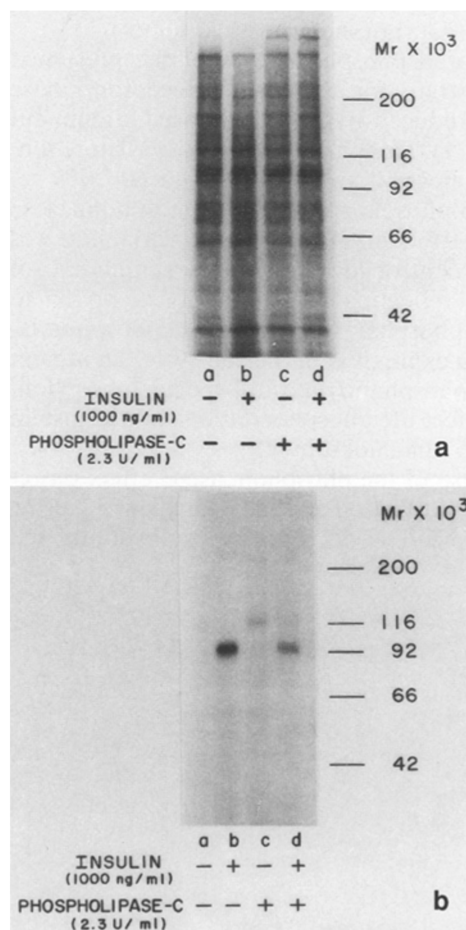

Fig. 6. Effect of phospholipase C on insulin-stimulated autophosphorylation of the β-subunit of the insulin receptor in intact NIH 3T3 HIR 3.5 fibroblasts. **Panel a** shows the phosphoprotein profile obtained from TCA (10%) precipitation of 30 μl of crude extract; **Panel b** is an immunoprecipitation of the same extract with anti-phosphotyrosine antibodies. Both preparations were resuspended in Laemmli buffer with 100 mmol/l DTT, boiled for 3 min, and the proteins were separated on SDS-PAGE, and identified by autoradiography

after insulin stimulation, no effect on levels of receptor autophosphorylation was observed (data not shown).

Discussion

In the present study, we have examined the effect of alterations of plasma membrane lipids on insulin action by digestion of cells with two different phospholipase C preparations. One phospholipase C is from *C. perfringens* and has a broad specificity, but acts primarily as a phosphatidylcholine phosphohydrolase. In general, this phospholipase C alters the polar head groups of membrane lipids [29, 37]. The other is a phosphatidylinositol-specific phospholipase C from *Staphylococcus aureus* which catalyzes exclusively the hydrolysis of glycosyl-phosphatidylinositol anchor [31]. Treatment of intact cells with *C. perfringens* phospholipase C, but not with the phosphatidylinositol-specific phospholipase C, reduced insulin-stimulated receptor autophosphorylation without affecting insulin binding. This inhibitory effect was observed within 10 min of treatment with phospholipase C, but was not observed in cells which had been first stimulated with insulin. Thus, the phospholipase C treatment disrupts the transmembrane signalling from the α- and β-subunit of insulin receptor but does not appear to reduce kinase activity that is established.

Phospholipase C readily hydrolyzes 40–60% of the phospholipids present in a variety of different biological membranes [29, 37]. This treatment can change the molecular environment around the insulin receptor which ultimately affects insulin receptor signalling [38]. Previous studies of insulin receptors reconstituted in vesicles of different lipid composition suggest that the membrane lipid environment, especially the degree of unsaturation of the phospholipid fatty acylchains, can influence the binding properties of insulin receptor [39]. The decreased levels in insulin-stimulated autophosphorylation observed in these experiments are due to altered signal propagation rather than to a direct action of phospholipase C on insulin receptor binding since, under our conditions, binding does not appear to be affected. Furthermore, this effect was due to the lipid hydrolyzing action of these enzymes and was inhibited by addition of phosphatidylcholine. Protease inhibitors did not eliminate the phospholipase C effect, ruling out a role for possible contaminating proteases in these observations. More importantly, when the insulin receptor was partially purified on WGA and the autophosphorylation assay was carried out in an in vitro system, there was no difference between treated and untreated cells. Thus, when the insulin receptor was reconstituted in an environment where most membrane lipids were replaced by detergent (Triton X-100), the effect of phospholipase C treatment was no longer present [40].

From these results, we conclude that the alterations in the lipid environment caused by phospholipase C are responsible for the effect on insulin receptor autophosphorylation. Exactly how changing the lipid environment might alter receptor kinase activation is uncertain. It is possible that the lipid environment plays an important permissive role in some change in receptor conformation or aggregation induced by insulin and required for normal signal transduction. Phospholipase treatment may also change the surface charge properties of the membrane and produce some electrostatically unfavourable environment for transmembrane signalling [41]. Indeed, it is possible that the phospholipase effect is not specific to the insulin receptor and also affects membrane receptors for other ligands such as the EGF or IGF-I receptors. Recent data in our laboratory (Huertas, P., Kahn, C. R., unpublished observation) in which receptors have been incorporated into a variety of different lipids suggest that the nature of the polar head group, as well as fatty acid chain length and unsaturation can have major effects on the ability of insulin to activate the receptor kinase.

An alterative explanation could be that exogenously added phospholipase C leads to an increased level of diacylglycerol in cells which in turn could activate protein kinase C [42, 43]. Protein kinase C can phosphorylate the insulin receptor on serine and threonine residues resulting in a decrease in tyrosine kinase activity [44, 45]. In our experiments, however, even after 40 min of treatment with phospholipase C, there was no change in the basal level of receptor phosphorylation as detected by immunoprecipitation with both anti-phosphotyrosine antibodies and anti-insulin receptor antibody. Furthermore, there is evidence that in other cell types incubation with phospholipase C causes a loss, rather than an increase, in protein kinase C activity in the particulate fraction [46].

Insulin-stimulated phosphorylation of pp 185, the putative substrate for insulin receptor kinase [47], was more markedly inhibited by phospholipase C treatment than that of receptor itself. Based on in vitro mutagenesis, the binding site for this substrate has been localized in the juxtamembrane region of the β-subunit of insulin receptor [48]. Thus, alterations in the lipid environment could either directly affect the interaction between insulin receptor and pp 185 or inhibit same change in conformation necessary to expose the substrate binding site. This point deserves further study.

Cuatrecasas showed that digestion of adipocytes and liver cell membranes with phospholipase C led to a three-six-fold increase in specific insulin binding with no change in glucagon binding [25]. In adipocytes this was accompanied by a loss in insulin-stimulated glucose oxidation, even at low concentrations of phospholipase at which the insulin binding was unchanged. In the present study, no increase in insulin binding was observed. This may be due to different phospholipase C preparations or to differences in membrane lipids in the different cells used. However, we did find a dramatic effect of phospholipase C on insulin receptor phosphorylation despite the presence of normal insulin binding. Thus, in both systems phospholipase C treatment resulted in a post-binding form of insulin resistance.

An alternative approach to study the effects of lipid modification on receptor function is to attempt to alter cell membrane lipid composition by culture of cells in media supplemented with different lipids. This technique has been used to show effects of lipids on both insulin binding and insulin action in 3T3-L1 cells [19], hepatoma cells [10] and a variety of other cell types [8]. Recently

Bruneau and colleagues [10] have studied insulin action in rat hepatoma cells whose membrane lipid composition was altered by supplementation of the culture medium with linoleic acid or 25-hydroxycholesterol. In this model, they found that the induced alteration in the lipid composition resulted in alterations on both binding and biological actions of insulin, creating an insulin resistant state, although the exact site of the resistance to insulin action remained uncertain.

In summary, our data suggest that phospholipase C treatment of cells affects insulin action by altering transmembrane signalling by the insulin receptor. Thus, membrane lipid composition may have profound effects on this earliest event in insulin action. Similarly, alterations of membrane lipid composition might be involved in insulin resistance states such as obesity or non-insulin-dependent diabetes mellitus [49–51]. Clearly, more studies of regulation of insulin receptor activity by lipids are needed to define the critical role these membrane components may play in insulin signal transduction, and how this may contribute to insulin resistant states.

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