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Characterization of an Insulin Receptor Serine Kinase (I R S K)

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ABSTRACT

Insulin acts by binding to a specific cell surface insulin receptor (IR) resulting in rapid stimulation of receptor tyrosine autophosphorylation and tyrosine kinase activity (TKA). The IR itself is also phosphorylated on serine in response to insulin. This reaction has been postulated to be catalyzed by an insulin receptor serine kinase (IRSK).

To characterize the IRSK activity, IR were partially purified from cultured IM-9 human lymphocytes. In vitro stimulation revealed that tyrosine phosphorylation preceded serine phosphorylation. In contrast, pre-activation of IR prior to assay by exposure of intact cells to insulin in vivo resulted in early detection of serine and tyrosine phosphorylation, peaking at 1 minute with a decrease after 30 to 60 minutes exposure. Serine phosphorylation was detected on only those IR which were tyrosine phosphorylated. Neither treatment with 1M NaCl nor immunoprecipitation of total IR before or after phosphorylation could dissociate the IR from IRSK activity.

IRSK activity was inhibited in direct proportion to tyrosine autophosphorylation by : genistein, a tyrosine kinase inhibitor : by lowering $MnCl_2$ concentration : and by phorbol-12-myristate-13-acetate (PMA).

Dilution of the IR preparation or addition of glycerol did not alter the ratio of phosphoserine to phosphotyrosine incorporation suggesting that both amino acids are phosphorylated via an intramolecular reaction.

Incubation of preactivated IR to allow maximal serine phosphorylation resulted in a decrease in TKA assessed with the exogenous substrate poly Glu:Tyr (4:1).

Our data suggest the novel possibility that the IR may autophosphorylate on serine subsequent to tyrosine phosphorylation. This serine autophosphorylation appears to be associated with diminished tyrosine kinase activity and may explain in part the well documented phenomenon of insulin-induced insulin resistance.

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Finally , I would like to thank my girlfriend, Caroline, for her encouragement and belief she gave me to complete this 2 year project and for the understanding and patience to write this thesis .

This is dedicated to the Rosemount Redskins and to the memory of a very close companion of mine , Brandy .

Without my sunshine and my moonlight , my life would not be complete .

Peter Nower

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INTRODUCTION

The peptide hormone, insulin (6000 Da) (Fig. I), secreted from the beta cells of the islets of langerhans in the pancreas, is a major regulator of glucose homeostasis in mammals (Nogrady, 1988). Thus increasing our knowledge about the regulation of insulin secretion and insulin action is important to understand the normal physiological and pathophysiological processes that lead to changes in carbohydrate metabolism and disease states such as diabetes mellitus (type I & II). The goal of this research study was to investigate one aspect of the regulation of insulin action.

1.1 Insulin Action

The mechanism by which insulin acts on its major target tissues: liver, muscle, and fat, is incompletely understood. For many years it was known that insulin altered protein, carbohydrate and fat metabolism by altering the activities of a variety of intracellular enzymes which convert the fuels or related substrates from one form to another (Czech et al., 1988; Zick, 1989). For example, glucose conversion to glycogen is stimulated by insulin via the activation of the enzyme glycogen synthase (Hollenberg, 1990). Research into the mechanism of action of pharmacological agents led to the concept of hormone action being mediated via binding to specific proteins or receptors. In the 1970's, evidence from various laboratories indicated

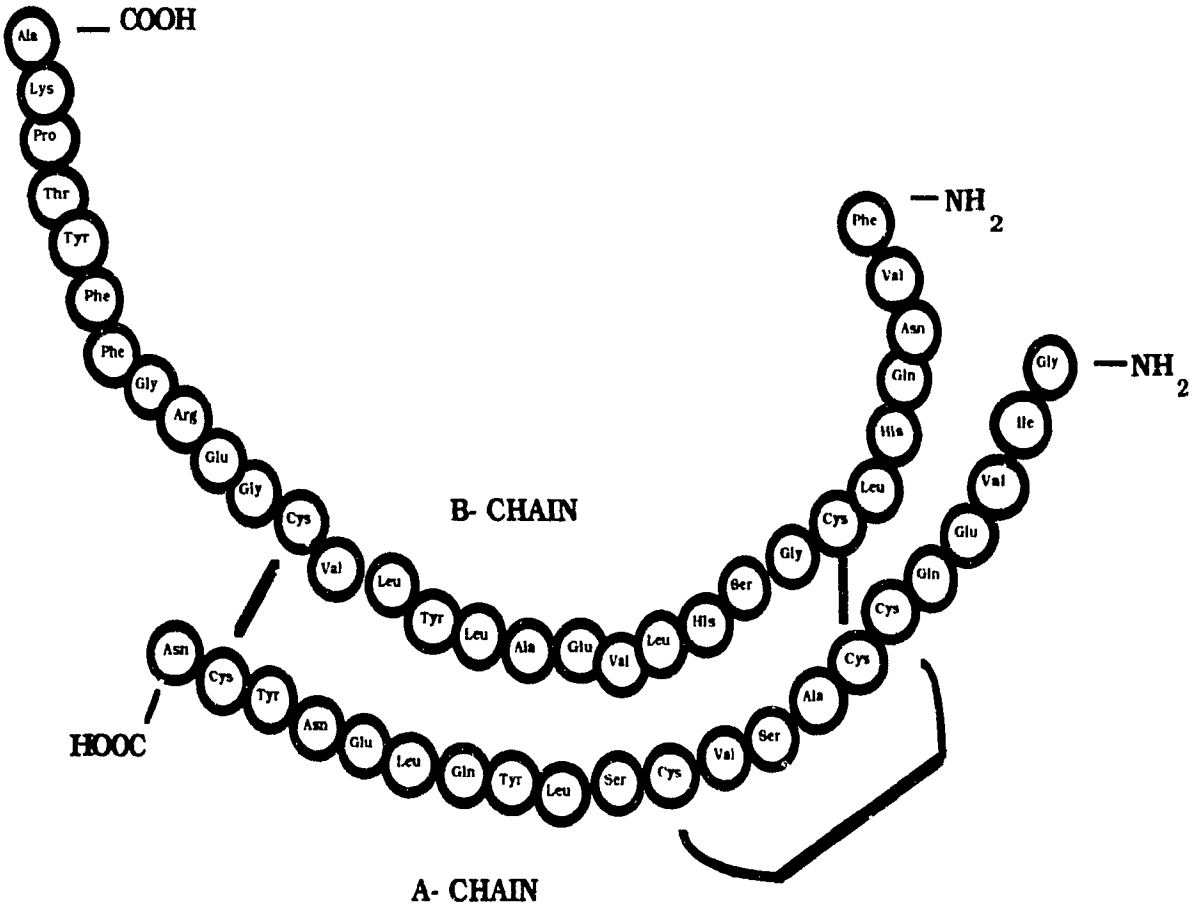


Fig. I Amino acid sequence of human insulin. The insulin molecule is made up of 2 peptide chains (A - chain - 21 amino acids long and a B - chain - 30 amino acids long.) which are connected by 2 disulfide bonds (black lines) and contains one additional disulfide bond within the A- chain.

that the first step in insulin action was the binding of hormone to a specific receptor located on the plasma membrane (Freychet et al., 1971; Cuatrecasas, 1972; Kahn, 1976 & 1985; Czech, 1984). Studies of covalent crosslinking of insulin to its receptor (Gammeltoft et al., 1984; Roth et al., 1981; Kahn, 1976) and eventually the cloning and sequencing of the insulin receptor cDNA led to the elucidation of its structural components (Ullrich et al., 1985; Ebina et al., 1985). The insulin receptor (350 kDa) is a cell surface heterotetrameric transmembrane glycoprotein (Kahn et al., 1985; Zick, 1989) with 2 α subunits (135 kDa on SDS-PAGE) totally external to the cell and 2 β subunits (95 kDa on SDS-PAGE) which span across the plasma membrane and enter the cytoplasm of the cell (Jacobs et al., 1977; Fugita-Yamaguchi et al., 1983; Van Obberghen et al., 1981; Hedo et al., 1981; Kasuga et al., 1981). These subunits are joined together by disulfide bridges to give a final tetrameric structure of the receptor (Fugita-Yamaguchi et al., 1984; Massague et al., 1982). The disulfide bond(s) linking the two α-α receptor halves are more sensitive to reduction than those linking the α and β subunits (Rosen, 1987) (Fig. II).

The α-subunit lacks a transmembrane domain and lies completely external to the plasma membrane (Zick, 1989). It contains the insulin binding domain and is glycosylated on asparagine residues to yield a high mannose form which appears to be necessary for IR to bind insulin (Forsayeth et al. 1986; Hedo et al., 1981; Ronnett et al., 1984; Kahn et al., 1988). The precise amino acid sequence and protein tertiary structure involved in this binding are the subjects of

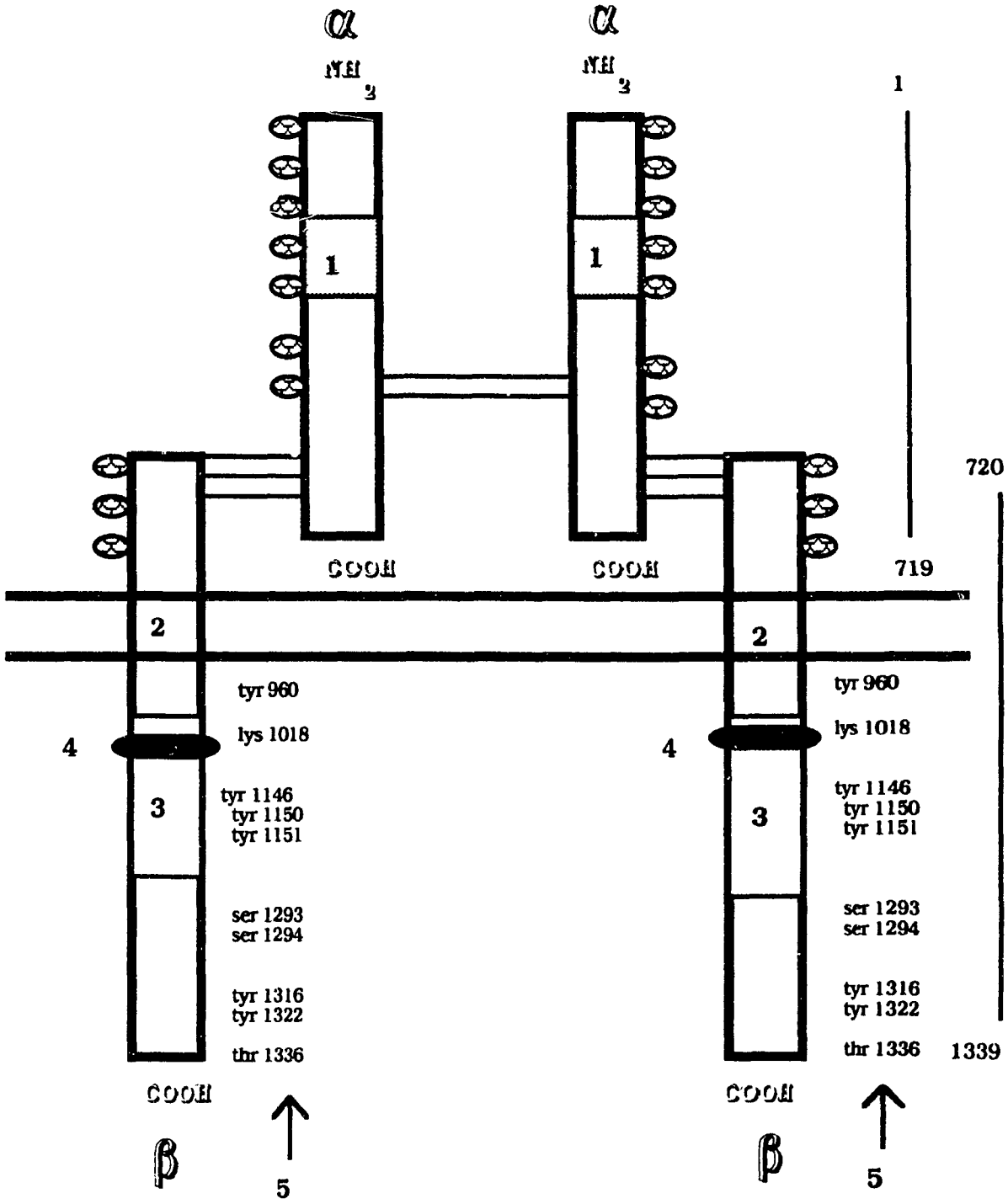


Fig. II Schematic depiction of the human insulin receptor. The extracellular domain contains cysteine rich areas (1), and is glycosylated at various points. The transmembrane region (2) extends internally to the cytoplasmic domain which contains the tyrosine kinase domain (3), the ATP binding site (4), and other amino acid residues important to the insulin receptor (5). The alpha and beta subunits are held together by disulfide bonds (▬) (numbering of amino acids according to Ullrich).

investigation in several laboratories. Another region of the alpha subunit is the cysteine rich region (refer to Fig.II). Made up of 16.5% cysteine and 72% hydrophilic residues (suggesting that this region is in a hydrophilic enviroment) , this rigid region accounts for the intra and intermolecular disulfide bridges needed to activate this $\alpha 2$-$\beta 2$ heterotetrameric receptor. This is common to various other cell surface receptors, and may be important in microaggregation of receptors stimulated by ligand binding (Posner et al., 1989; Baron et al., 1990; Kahn et al., 1978; Isersky et al., 1978; Schechter et al., 1979).

The β-subunit contains a transmembrane domain which anchors the receptor in the membrane (Sabatini et al., 1982). The early cytoplasmic portion contains a region important for internalization (Chen et al., 1990). This N-P-X-Y (where X stands for any amino acid and Y represents tyrosine 960) domain is found in other cell surface receptors in which mutations have been associated with impaired receptor mediated endocytosis (Chen et al., 1990; Backer et al., 1990).

A major region of the β-subunit cytoplasmic domain contains a tyrosine kinase domain with homology to other nonreceptor and receptor tyrosine kinases such as $pp60^{v\text{-}src}$, the platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and insulin like-growth factor one (IGF-1) receptor (Hanks et al., 1988). This consists of an ATP binding domain characterized by a glyXglyXXgly followed by a lysine at position 1018 (Kahn et al. , 1978; Heffetz et al., 1986; Boni-

Schnetzler et al., 1986; Ebina et al., 1987; Chou et al., 1987) (numbering according to Ullrich et al., 1985) as well as the catalytic region (Kasuga et al., 1982a Gammeltoft et al., 1986; Rosen, 1983, Goldfine , 1987; Kahn et al., 1988; Van Obbergan et al., 1983; Pilch et al., 1983). A regulatory domain has been intensively investigated consisting of 3 tyrosine residues at positions 1146, 1150, and 1151 (Ullrich et al., 1985).

A number of studies of both in vivo and in vitro receptor stimulation by insulin revealed that phosphorylation of these regulatory tyrosine residues occurs within seconds after insulin binding (Zick et al., 1983a; Roth et al., 1983; Kasuga et al., 1982b & 1982c; Haring et al., 1982; Kasuga et al., 1983, Petruzzelli et al., 1982). In order for tyrosine phosphorylation to take place upon insulin binding to the IR alpha subunit , some sort of conformational change must take place within the extracellular domain of the receptor. The signal is then transmitted down the axis of the molecule until it reaches the cytoplasmic domain (Posner et al., 1989; Baron et al., 1990). This autophosphorylation reaction results in the activation of the receptor as a tyrosine protein kinase which is able to actively phosphorylate exogenous substrates on tyrosine. Examples of substrates used to measure such activity include histone, angiotension and the artificial substrate peptide poly Glu:Tyr (4:1) (Kasuga et al., 1983; Zick et al., 1983; Stadtmauer et al., 1983). In this activated tyrosine autophosphorylated state of the receptor, the insulin may be removed from the receptor without loss of activity (Braum et al., 1984; Zick et al., 1985). Presumably other factors such as dephosphorylation of

tyrosine are required to reduce activity to basal. Other tyrosine sites are also phosphorylated upon insulin stimulation. These include 2 residues in the carboxyl-terminal region tyrosine 1316 and 1322 (Tavare et al., 1988 & 1990). The function of this phosphorylation is not clear. Augmentation of tyrosine kinase activity does not result, and some workers have postulated a paradoxical dampening of receptor enzyme activity but this remains unproved. Nevertheless, since receptor tyrosine residues are generally not completely occupied by in vivo activation of receptor by insulin, (i.e. exposure of intact cells or tissues to hormone), pre-activated receptors usually demonstrate augmented autophosphorylation activity as well as augmented tyrosine kinase activity measured with exogenous substrate (Rosen et al., 1983; Yu et al.: 1984 & 1986; Kwok et al., 1986; Klein et al., 1986; Stadtmauer et al., 1986; Herra et al., 1986; Pang et al., 1985; Kohanski et al., 1986; Morrison et al., 1987; Tornqvist et al., 1988).

The mechanism of the stimulation of tyrosine autophosphorylation by insulin is not clear. Recent data indicates that a transphosphorylation recation occurs in which one β-subunit "cross-phosphorylates" its disulfide linked neighbour (Honegger et al., 1989 & 1990; Ballotti et al. 1989; Boni-Schnetzler et al., 1988; Feitz et al., 1988). Experiments with α-β monomers indicate that insulin stimulates dimerization and autophosphorylation (Sweet et al., 1987). In a dilute solution, α-β dimers do not undergo autophosphorylation while heterotetramers do, indicating the intramolecular nature of the cross - phosphorylation reaction (Sweet et al., 1987). Recently, it has been demonstrated that in a very concentrated solution, greater than

or equal to 60 μg of insulin receptor/ml, there may be a small amount of intermolecular phosphorylation that takes place (Hayes et al., 1991). Whether this ever occurs under normal conditions in vivo is not clear, but it may have relevance to studies in which receptor cDNA is transfected into cells that greatly overexpress these proteins (Soos et al., 1989).

The role of insulin induced receptor internalization in the transduction of insulin's signal is not clear. Since the receptor β-subunit is internalized as an activated tyrosine kinase and is cytoplasmically orientated, the possibility of phosphorylation of intracellular substrates exists. Although a recent study suggests that hormone-receptor complex internalization is not required for insulin action (McClain et al., 1987; McClain, 1990), this remains to be confirmed.

The carboxy-terminal region may serve two functions. Some evidence suggests that it is essential for transmitting the signal for specific metabolic biological responses as opposed to the growth effects of insulin (Olefsky, 1990). This region may be required for binding of some signalling molecules. Secondly, it has been noted that serine and threonine residues in this region may also be phosphorylated under certain conditions. At least in some cases, this has been associated with diminished tyrosine kinase activity and insulin resistance (Lewis et al., 1990a, 1990b).

Since the structural and at least some of the functional properties of the receptor are known, it has been suggested that autophosphorylation and activation of the receptor tyrosine kinase is essential for insulin action. The evidence for this conclusion is based upon the following observations:

1) The correlation of receptor kinase deficiency with insulin resistance. This has been observed in mutant receptors in genetic disorders associated with insulin resistance in man (Zick et al., 1989), synthetic mutant receptors expressed in Chinese Hamster Ovary cells (CHO cells) and other cells which lack tyrosine kinase activity, e.g. mutation of lysine 1018 to alanine (Maegawa et al., 1988 ; McClain et al., 1987; Chou et al., 1987; Ebina et al., 1987) , and diminished tyrosine kinase activity in animals and man in acquired states of insulin resistance such as obesity (Zick et al., 1989).

2) The ability of anti-receptor antibodies which inhibit tyrosine kinase activity to inhibit insulin action when introduced into target tissues (Gammeltoft et al., 1986).

3) The correlation of activation of the insulin receptor kinase with stimulation of insulin biological effects by insulin-mimetic agents such as hydrogen peroxide (Czech et al., 1974; May et al., 1979; Little et al., 1980; Heffetz et al., 1989 & 1990), vanadate (Tamura et al., 1983a & 1984a; Heffetz et al., 1990; Fantus et al.,

1990), trypsin (Tamura et al., 1983b & 1984b) and peroxide(s) of vanadate (Kadota et al., 1987; Fantus et al., 1989).

The best evidence for requirement of the receptor tyrosine kinase for biological response will be the discovery of an endogenous cellular substrate. Upon tyrosine phosphorylation of the substrate, the insulin receptor would transmit a response such as enzyme activation or protein translocation. At this point, two such substrates have been identified: the enzyme phosphatidylinositol-3-kinase and insulin receptor substrate one (IRS-1). IRS-1 is a protein that has recently been cloned by Sun et al. (Sun et al., 1991) which contains multiple tyrosine phosphorylation sites and may function to bind signalling molecules. The precise role of these proteins in mediating the varied actions of insulin remains to be determined.

A known consequence of insulin signalling is the phosphorylation and dephosphorylation (Hollenberg et al., 1990) of various proteins on serine/threonine residues (Avruch et al., 1985). Several laboratories have provided evidence for the activation of serine kinases which may mediate these events (Gazzano et al., 1983; Yu et al., 1987a ; Smith et al. , 1988a ; Price et al. , 1990). It has been proposed that the insulin receptor may activate one or more serine kinases by tyrosine phosphorylation, and that a cascade of serine kinases may follow to mediate the final biological responses (Czech et al., 1988). Some evidence for this has been found in several studies demonstrating the activation by insulin of various serine kinases (Gazzano et al., 1983; Yu et al., 1987b; Klarlund et al., 1988; Ray et al.,

1988a). Of particular interest was the purification of microtubule associated protein 2 (MAP-2) kinase, named after its substrate by Ray and Sturgill (Ray et al., 1988a&b). This enzyme was found to be activated by insulin and phosphorylated on tyrosine (Ray et al., 1988c ; Boulton et al., 1988). More recent studies have revealed that full activation requires tyrosine and threonine phosphorylation (Anderson et al., 1990), and that it appears to be one of a family of similar enzymes. One of these enzymes, designated extracellular signal regulated kinase (ERK-1), containing potential tyrosine phosphorylation sites has been cloned by Cobb (Boulton et al. , 1990 ; Erikson et al., 1990) . This has led to the change in nomenclature to mitogen activated protein (MAP) kinase. MAP Kinase is itself a serine/threonine kinase and may activate other kinases such as one form of ribosomal protein S6 kinase (Sturgill et al., 1988). Of interest, these initial receptor-activated serine/threonine kinases such as MAP kinase and other mammalian (Ben-David et al., 1991) and yeast homologues (Featherstone et al., 1991) may phosphorylate themselves and/or exogenous substrate on tyrosine (see Discussion).

Finally, aside from the kinase cascade, another mechanism of insulin signalling has been proposed. The activation of a phosphatidylinositol specific phospholipase C, cleaves a putative second messenger molecule, an inositol phosphate glycan (IPG), from phospholipid (Larner , 1988; Saltiel et al., 1986a, 1986b, 1987a, 1987b). This cleavage also yields diacylglycerol (DAG), an activator of PKC (Larner, 1988). This IPG has been found to stimulate glycogen synthase and pyruvate dehydrogenase (Saltiel, 1990; Czech, 1985;

Belfrage et al., 1986). Both of these enzymes are activated by insulin via dephosphorylation which suggests that the IPG may activate protein phosphatase. The mechanism by which the insulin receptor activates the phospholipase C is not known, but it does not appear to be via tyrosine phosphorylation (Nishibe et al., 1990). A change in receptor conformation leading to interactions with intermediary proteins such as GTP binding (G) proteins has been suggested but remains to be demonstrated.

In summary, the mechanism of insulin signalling involves: binding of insulin to its receptor, stimulation of an autophosphorylation reaction of the β-subunit on tyrosine and activation of the enzyme as a tyrosine protein kinase. The subsequent biochemical events remain uncertain but appear to involve more than one pathway and involve the activation of kinases and phosphatases.

1.2 Insulin Resistance and Receptor Serine Phosphorylation

The physiological regulation of insulin action sometimes results in resistance to insulin, e.g. during fasting. Our knowledge of insulin action would predict that the number or affinity of insulin receptors available to bind hormone at the cell surface and/or their ability to undergo autophosphorylation and activation of the tyrosine kinase may be modulated and result in impaired biological response. The example of prolonged fasting in rats has been associated with a diminished ability of insulin to stimulate receptor phosphorylation. This may be associated with inhibition of tyrosine kinase activity (Karasik et al.,

1990). Previous studies utilizing the phorbol ester, phorbol-12-myristate-13-acetate (PMA), which is a diacylglycerol analogue that activates the Ca^{++} and phospholipid-dependent protein kinase C (Castagna et al., 1982), demonstrated that in various cell types PMA induces insulin resistance with a concomitant stimulation of insulin receptor serine and threonine phosphorylation (Takayama et al., 1984, 1988; Haring et al., 1986a; Lewis et al., 1990a). This resistance was associated with an impairment in insulin stimulation of receptor autophosphorylation and tyrosine kinase activity (Takayama et al., 1984; Karasik et al., 1990). This phenomenon has also been suggested to occur upon exposure of cells to isoproterenol, a beta receptor agonist which activates cAMP-dependent kinase or protein kinase A (Haring et al. 1986b). In vitro experiments demonstrated that purified protein kinase C (PKC) can phosphorylate the purified insulin receptor and this results in decreased tyrosine kinase activity (Bollag et al. , 1986).

Insulin resistance is also induced by exposure to insulin itself. As well, a few studies have documented that insulin incubated both in vitro with partially purified insulin receptors and in intact cells in vivo can stimulate the phosphorylation of the insulin receptor on serine, and in some experiments, threonine. Recent investigation of this phenomenon by Lewis et al revealed that the sites of phosphorylation are in the carboxy-terminal region, serine 1293 and/or 1294 and threonine 1336 (Lewis et al., 1990a&b). Of interest, the same group reported that these same amino acids were phosphorylated in response to PMA (Lewis et al., 1990a) raising the possibility that

either protein kinase C was activated by insulin (which has been suggested by others (Duronio et al., 1990) or that another serine kinase, an insulin receptor serine kinase (IRSK), could be activated by insulin as well as by protein kinase C (Lewis et al. , 1990a). Of interest, Koshio recently reported in abstract form that serine number 1308 and/or 1309 was phosphorylated in the presence of PMA (Koshio et al., 1991). It may be that different types of stimuli, time or cell type result in the variability of serine phosphorylation.

A number of investigators have postulated the existence of an IRSK (Insulin Receptor Serine Kinase) (Kasuga et al., 1982a; Zick et al., 1983a; Lewis et al., 1990b; Smith et al., 1988a; Tavere et al., 1991; Gazzano et al., 1983; Biener et al., 1990). Studies with IM-9 lymphocytes have demonstrated that a serine kinase was activated by insulin in the wheat germ purified fraction using an exogenous substrate, histone (Balloti et al. , 1986) . A putative IRSK has been studied by Lewis et al. (Lewis et al., 1990b). This enzymatic activity could be stimumated by insulin, both in vitro and in vivo, but the mechanism has not yet been defined. Lewis demonstrated that phosphorylation of a peptide substrate resembling the carboxy-terminal region of the receptor is stimulated by insulin added to the receptor preparation (Lewis et al., 1990a). Therefore it does not appear to be merely an alteration in receptor conformation induced by insulin, which then permits an already active IRSK to phosphorylate on serine.

The IRSK may be one of the important serine/threonine kinases involved in regulating insulin action. Activation of a putative IRSK by insulin resulting in serine phosphorylation of the IR β-subunit may inhibit activity and thus play a role in a negative feedback system.

The goals of these studies were to characterize the activation and behaviour of the putative IRSK, and to begin to determine the biochemical consequences of insulin-stimulated insulin receptor serine phosphorylation to define the role of this action of insulin in cell biology. We provide evidence for a novel concept, the possibility that the IRSK may be the insulin receptor itself and that insulin stimulation of tyrosine autophosphorylation activates the receptor serine kinase activity. As well, our data are consistent with an inhibition of insulin receptor tyrosine kinase activity as a consequence of the insulin stimulated serine phosphorylation. These data provide a model for the mechanism of insulin-induced homologous desensitization of its own receptor.

MATERIALS AND METHODS

2.1 Chemicals purchased

Various chemicals were purchased from the following companies:

Anachemia (Montreal,Quebec) : Sodium Chloride, Magnesium Sulfate (Anhydrous), Sodium Bicarbonate, Sodium Phosphate (dibasic), Methanol, Sodium Pyrophosphate, Glycerol, Sodium Hydroxide, Acetic Acid, Potassium Hydroxide, Pyridine, Ether, Acetone, Trichloroacetic Acid.

Sigma (St.Louis,Missouri): 4-(2-Hydroxylethyl)-1-piperazine-ethanesulfonic Acid (Hepes), Triton X-100, Bovine Serum Albumin (BSA-4374), Ethylenediaminetetraacetic Acid (EDTA), Aprotinin, Sodium Fluoride, Sodium Orthovanadate (Na_2VO_3), Benzamidine, Phenylmethylsulfonyl Fluoride (PMSF), Leupeptin, Pepstatin A, N-Acetyl-D-Glucosamine (NADG), Rabbit γ-Globulin, Sodium Molybdate, Trizma Base, Phorbol-12-Myristate-13-Acetate (PMA), Dowex AGI-X8 (Cl Form), Tween-20, Phosphoserine, Phosphothreonine, Phospho-tyrosine, Sigmacote, Ponceau Red, Goat Anti Rabbit - Antibody (GAR), Anti-Phosphotyrosine Antibody .

Fisher Scientific (Montreal,Quebec): Potassium Chloride, Potassium Phosphate (dibasic), Dimethyl Sulfoxide, Polyethylene Glycol (PEG) (8000), Manganese Chloride, Hydrogen Chloride, Whatman Chromatography Paper (3mm), Falcon Tissue Culture Flasks (175cc).

Bio-Rad (Mississauga,Ontario): Sodium DodecylSulfate (SDS), Low & High Molecular Weight Standards, 2-Mercaptoethanol, Glycine, Acrylamide, Coomassie Blue, Bromophenol Blue, N,N,N',N'-Tetramethylenediamine (TEMED), Dithiothreitol (DTT), Ammonium Peroxysulfate (AF), N,N'-methylene- bis-acrylamide (BIS).

Gibco Canada Inc. (Burlington, Ontario): RPMI-1640 Medium, Fetal Bovine Serum (FBS), Antibiotic-Antimycotic (100X) (10000 units penicillin G/ml, 10000µg streptomycin sulfate/ml, 25µg amphotericin B/ml all in 0.85% saline; spectrum: bacteria, fungi, yeasts), Genistein.

Pharmacia LKB Biotechnology Inc. (Sweden) : Wheat Germ Agglutinin Sepharose, Protein A Sepharose .

BDH Chemicals (Ville St.Laurent , Quebec): Ammonium Hydroxide, Ninhydrin, Glutaraldehyde, Cellulose Coated Plastic Thin Layer Chromatography Plates (20 x 20 cm.; Thickness, 0.1 mm).

Boehringer Mannheim (Dorval, Quebec): Adenosine 5'-Triphosphate Disodium Salt.

Amersham Corporation (Oakville, Ontario): Carrier free Sodium ^{125}Iodide (350-600mCi/ml).

Du Pont-New England Nuclear (Mississauga, Ontario): [γ-^{32}P] ATP (3000Ci/mmol).

Commercial Alcohols Limited (Montreal, Quebec): Ethanol.

American Type Culture Collection (Rockville, Maryland): IM-9 Cells.

Picker International Canada (Montreal, Quebec): Kodak X-Omat Film.

Millipore (ST.Laurent, Quebec): Nitrocellulose Paper.

Aldrich Chemical Company (Milwakee, Wisconson): Paraffin Oil.

Pierce (Rockville, Ontario): Constant Boiling HCl (6N).

Glace Seche Quebec INC. (Montreal, Quebec): Dry Ice.

Steinberg Inc. (Montreal , Quebec): Carnation powdered milk.

The following were gifts: Anti Insulin Receptor 960 Antibody (an antibody raised against the 960 region of the β-subunit of the insulin receptor) was a generous gifts from Dr. Barry I. Posner (McGill University, Montreal, Quebec), Genistein was a generous gift from Dr. Pierre Laneuville (Royal Victoria Hospital, Montreal, Quebec, Canada) and monocomponent porcine insulin was kindly provided by Eli Lilly Co. (Indianapolis, Indiana).

2.2 Cell Culture

Human lymphocytes (IM-9) were cultured in Falcon tissue culture flasks (175 cc) with sterile and filtered RPMI-1640 supplemented with 25 mM HEPES (pH 7.6), 24 mM $NaHCO_3$, 1% antibiotic-antimycotic and 10% FBS at 37°C in a humidified atmosphere of 25% CO_2 and air as described previously (Fantus et al.,1982). Cells in late log phase were harvested by centrifugation at 1000 RPM for 10 minutes at (4°C) and washed twice with phosphate buffer saline (PBS) (2.68mM KCL, 1.47mM KH_2PO_4, 136 mM NaCl, 8.06 mM Na_2HPO_4-$7H_2O$, (pH 7.6)). They were resuspended in fresh RPMI-1640, aliquoted into 50ml conical tubes at a concentration of approximately 10^8cells/ml and incubated at 37°C with or without porcine insulin (10^{-7}M) and\or with or without PMA (5 & 100 nM) for the various times indicated.

2.3 Cell Solubilization

Immediately following the treatment, the incubation was terminated by addition of an ice cold (4°C) solubilization buffer (50mM HEPES (pH 7.6), 1% Triton X-100, 4mM EDTA, 1 trypsin inhibitor unit (TIU)/ml aprotinin, 25mM NaF, 1mM NaV, 1mM benzamidine, 1mM PMSF, 20 μM leupeptin, 20 μM pepstatin A) immediately frozen in dry ice and ethanol to -70°C. After at least 1 hour, the samples were thawed and homogenized in a Potter-Elvejem type homogenizer. The samples were vigourously shaken for 1 hour at 4°C and the solubilized extracts were centrifuged at 100,000 X g for 1 hour at 4°C (Beckman SW 60 Ti rotor) to remove unsolubilized material.

2.4 Partial Receptor Purification

The 100,000 X g supernatant was applied to a column (0.3 x 10 cm) of wheat germ agglutinin (WGA)-sepharose 6 MB (recycled 5 times) at 4°C and after washing with 100 mls of 4°C buffer (50 mM HEPES (pH 7.6), 150 mM NaCl, 0.1% Triton X-100), the bound material was eluted with the above buffer supplemented with 0.3M NADG, 1 Tiu/ml aprotinin , 1mM PMSF , 2mM NaF , 100 μM Na orthovanadate (Na_2VO_3) and stored at -70°C.

2.5 ^{125}I-Insulin Binding to Solubilized Cell Extract

For ^{125}I- insulin binding to solubilized receptors, the partially purified fractions (50 µl) were incubated for 20 hours at 4°C with ^{125}I - insulin (0.2ng/ml), iodinated as previously described (Fantus et al., 1982) in binding buffer (150mM NaCl, 50mM HEPES (pH 7.6), 10mM $MgSO_4$, 0.15% BSA) with or without 0.1-10000 ng/ml unlabeled insulin in a total volume of 0.3 mls. Final Triton X-100 concentration in the binding assay was 0.016 %. The assay was terminated by addition of 1 ml of 0.125 % rabbit γ - globulin and 1 ml of 25 % PEG both in 50 mM HEPES (pH 7.6) at 4°C followed by centrifugation at 2,000 X g for 30 minutes. The supernatants were aspirated, and the pellets were counted. Percent specific binding was then calculated by subtracting non-specific binding in the presence of 10 µg/ml unlabeled insulin from total binding and dividing that result by the total number of counts.

2.6 Insulin-Receptor Autophosphorylation

Aliquots containing equal amounts of receptor (350 - 450 fmoles) were added to 1.5ml eppendorf tubes to a "reaction buffer" containing final concentrations of 50mM HEPES (pH 7.6), 10 mM $MnCl_2$, 10 µM Na orthovanadate and 250 µM NaF in a final volume of 90 µl . The receptor was preincubated with or without 10^{-7} M insulin for 1 hour at 4°C. Phosphorylation was initiated by adding [γ-^{32}P] ATP at a final concentration of 25 µM (80 µCi/lane). The phosphorylation reaction was allowed to proceed at 22°C for 60

minutes, unless otherwise indicated, and terminated by adding 50 µl of stopping buffer (50mM HEPES (pH 7.6), 50 mM EDTA-$2H_2O$, 10mM Na Pyrophosphate, 1mM benzamidine-$1H_2O$, 20mM Na Molybdate- $2H_2O$, 0.1% Triton X-100, 40 mM NaF, 1% BSA, 1 mM PMSF, 2mM Na orthovanadate). It should be noted that in vitro activation of IR autophosphorylation denotes that insulin (10^{-7} M) is added directly to the partially purified IR preparation while in vivo activation refers to the addition of insulin (10^{-7}M) to intact lymphocytes at 37°C in culture for the times indicated and subsequent partial purification of the IR which are then autophosphorylated in vitro .

2.7 Immunoprecipitation

Ten µl of anti-human insulin receptor antibody (HIR) were added and the mixture shaken overnight for 16 hours at 4°C. The phosphorylated insulin receptor - HIR complex was then immunoprecipitated by incubating it with shaking with protein A sepharose for 1 hour at 4°C followed by centrifugation. The immunoprecipitate was washed once and microfuged with 50mM HEPES (pH 7.6), 0.1% SDS, 1% Triton X-100 and then twice with 50mM HEPES (pH 7.6) and 1% Triton X - 100.

2.8 SDS - Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The washed immunoprecipitate was suspended in 50 µl of "sample buffer" (final concentrations of 0.003% bromophenol blue,

15mM Tris, 6.9% SDS in Tris, 30% glycerol, 0.1M DTT, 15mM EDTA, 0.12M NaF, 0.03M Na pyrophosphate after dilution to 130 µl with water). Samples were then boiled for 5 minutes, centrifuged and equal volumes were subjected to SDS-PAGE (1.65% stacking and 7.5% resolving gel) under reducing conditions as described previously (Khan et al.,1986). Samples were run at 45 milli-amperes for 2.5 hours. After the migration of proteins was terminated, the gels were removed from the glass plates and destained (7% acetic acid and 10% methanol) for 40 minutes. The gels were fu ' ered prepared for drying by submerging them for 30 minutes in a destaining solution that included 3% glycerol. The gels were dried (30 minutes) and prepared for autoradiography. Phosphorylated receptor was visualized by autoradiography at -70°C using enhancing screens and exposed for 1 to 8 hours to Kodak X - Omat AR films.

2.9 Development and analysis of films

Films were removed from their casettes, loaded into film holders and submerged in Kodak developing solution for 4 minutes. They were then removed from the developing solution and submerged in running water for 2 minutes. Once washed, they were submerged in Kodak fixing solution for 4 minutes and later rinsed in running water for 5 minutes. They were finally dipped in a Kodak coating solution and dried. The films were then loaded onto a LKB ultrascan XL enhanced laser densitometer interfaced to an IBM-386 computer using the program GEL SCAN XL (Pharmacia INC.) to assess intensity of the 95-kDa band (located with molecular weight standards run

along side of the samples in the gel). The relative intensity of the 95 kDa band was quantitated by computer analysis of the area under the curve and is expressed in arbitrary units.

2.10 KOH treatment of SDS Polyacrylamide Gels

To remove labile serine and threonine phosphorylation, gels were destained with 7% acetic acid and 10% methanol overnight and then transferred to a 10% glutaraldehyde solution for 30 minutes. The gels were transferred to a solution of 1 M KOH at 56°C for 2 hours. The KOH solution was delicately removed and was replaced with a 10% acetic acid wash which was performed twice (2 X 2 hours). This procedure was performed before autoradiography (Cooper et. al.,1981) where indicated.

2.11 Phosphoamino Acid Analysis of the Insulin Receptor Beta Subunit

Phosphoamino acid analysis was performed with some modifications of the method of Cooper et. al.,1983. Following SDS-PAGE, the gels were fixed for 30 minutes in 30% methanol, 2% glycerol and dried. The radiolabeled 95-kDa band was excised and homogenized in 10 mls of 0.1M NH_4HCO_3 and 0.1% triton X-100 using a Potter-Elvejhem type homogenizer. To precipitate the protein, 5% of 2-mercaptoethanol and 5 µg/ml of rabbit γ - globulin (used as a carrier) was added to the homogenized gel and incubated together for 1 hour at room temperature. This mixture was then

centrifuged at 12,000 R.P.M to remove the gel. The supernatant was aspirated, placed in siliconized 2 ml Eppendorf tubes using Sigmacote and the phosphorylated protein was precipitated with 10% TCA. The precipitate was then washed with an ethanol and an ethanol/ether solution (1:1) and left at -70°C for 1 hour. The protein was acid-hydrolysed at 100°C for 3 hours using 6N HCl. The acid treated phosphoamino acids were then washed twice with water. The liberated [^{32}P] phosphoamino acids were purified from the hydrolysate by using an ion exchange, resin Dowex AG1-X8, at a pH of 7.5 to 8.5 for 4 hours. The resin was washed 3 times using 1 ml of water and the phosphoaminoacids eluted with 0 1M HCl for 20 minutes. These samples were microfuged and the HCl washes were saved in a 2 ml sigmacote treated eppendorf tube. The sample was dried using a speedvac. The [^{32}P] phosphoamino acids were then resuspended in 10 µl of water and loaded onto a precoated cellulose plastic sheet. This sheet was laid upon a paraffin oil coated thin layer electrophoresis (TLE) cold glass plate and the labelled amino acids were resolved by using a solution (H_2O, acetic acid and pyrimidine; 189:10:1) for 60 minutes at 1000 volts as described by Cooper et. al. (Cooper et al., 1983). Radioactive amino acids were visualized by autoradiography and identified by comparing to standards visualized with 0.2% ninhydrin in acetone.

2.12 Western Blotting Analysis

Phosphoproteins from SDS gels were transferred to nitrocellulose membranes by electrophoresis. The transfer was

carried out overnight at 4°C at 200 milli-amperes in a buffer containing 20% methanol, 192 mM glycine and 25 mM Tris- HCl (pH 8.8). Following the overnight transfer, the nitrocellulose was treated immediately with ponceau red to see if transfer of the proteins occurred and to visualize the molecular weight standards used to locate the 95-kDa band (β subunit of the insulin receptor). To determine phosphotyrosine content, the nitrocellulose membrane was washed with water and blocked with 50 ml of PBS (pH=7.4) containing 10% FBS shaking slowly for 1 hour at room temperature. The blocking solution was then removed and replaced with 50 mls of 1:300 diluted affinity purified anti-phosphotyrosine antibody in PBS (pH 7.4) containing 2% BSA. The incubation was shaken for 2 hours at room temperature. The antibody solution was removed from the nitrocellulose membrane, which was washed 3 times with a PBS solution (pH 7.4) containing 1% tween-20 for ten minutes. After these washes the blots were incubated with 50 mls of ^{125}I - goat anti-rabbit antibody, (^{125}I - GAR), ($0.7X10^6$ cpm / electrophoretic lane transferred) diluted in PBS (pH 7.4) containing 2% BSA for 1 hour at room temperature. The labeled antibody was aspirated and the nitrocellulose was again washed as before with PBS (pH 7.4) and 0.1% tween-20. The membranes were air dried and fixed to Whatman 3 mm paper. Labelled phosphotyrosine-containing proteins were visualized by autoradiography at -70°C using enhancing screens and Kodak X-Omat AR films. The intensity of labelling of the β subunit (95-kDa band) was quantified by densitometry as described above.

Assessment of receptor content in the gels was accomplished by immunoblotting with antibodies against the 960 region of the β subunit of the insulin receptor. The immunoblots were performed as described above for anti-phosphotyrosine antibodies except for the following minor modifications; proteins transferred to nitrocellulose paper were blocked with PBS (pH 7.4) containing 3% Carnation milk and then incubated for 2 hours at room temperature with a 1:300 dilution of anti-960 antibody in PBS (pH 7.4) containing 3% Carnation milk. The second antibody (^{125}I-GAR) incubation was also done in a suspension of PBS (pH 7.4) containing 3% Carnation milk.

2.13 Insulin Receptor Tyrosine Kinase Activity

Insulin receptor (390 fmol of insulin binding) partially purified using wheat germ agglutinin was first incubated with or without 10^{-7} M insulin for 60 minutes at 4°C in a reaction buffer as described above. Phosphorylation of the IR was initiated by the addition of unlabeled 25 μM ATP at 4 or 22°C and allowed to proceed for various times (0-120 minutes). The phosphorylation reaction of the exogenous substrate was initiated by addition of aliquots of the phosphorylated receptor to 2 mM $MnCl_2$, 15 mM MgSO4, 2.5mg/ml poly(Glu/Tyr) (4:1), and 7-8 μM [γ-^{32}P] ATP (5 μ Ci/tube) in a total volume of 160 μl as described previously (Kadota et al. , 1987 ; Zick et al. , 1983). After further incubation for 30 minutes at 4 or 22°C, the reaction was terminated by spotting 80 μl of the reaction mixture onto Whatman 3 mm filter paper which was placed into 10% TCA containing 10mM sodium pyrophosphate. After being extensivly washed in the above solution,

the paper was dried and placed in scintillation vials which contained 15 mls of Aquasol-II in which radioactivity was determined by β-scintillation counting (LKB , Model 1219).

2.14 Calculation of % phosphotyrosine and phosphoserine from the phosphoamino acid analysis

Films were scanned as described above. Areas of both regions representing phosphotyrosine and phosphoserine were added together. The areas representing either phosphotyrosine or phosphoserine were divided by the sum of the total area representing the two amino acids and multiplied by 100.

2.15 Calculation of total phosphotyrosine and phosphoserine content of the phosphorylated insulin receptor

% Phosphotyrosine and % phosphoserine were calculated as above. These values for % phosphotyrosine and % phosphoserine were then multiplied by the quantity of total phosphorylation determined by the densitometer in the 95 kDa band.

2.16 Reasons for choosing IM-9 Lymphocytes

Cultured human lymphocytes (IM-9's) have been used for many years as a model to study insulin receptor regulation. The down regulation of the insulin receptors by insulin was first demonstrated in this cell line by Gavin in 1975 (Gavin et al., 1975) and the stimulation

of insulin receptor phosphorylation by insulin was first demonstrated in these cells by Kasuga (Kasuga et al., 1982b & 1983c). The following summarize our rationale for using these cells to study insulin receptor serine kinase: 1) They contain a large number of insulin receptor (20000 receptors/ cell) which possess insulin stimulated autophosphorylation and tyrosine kinase activity. 2) They grow quite quickly in culture so that the large number of IR required for these studies would be available. 3) These cells can be easily treated in vivo with various substances (e.g. insulin and PMA) in a controlled fashion (e.g. time and temperature).

RESULTS

3.1 Serine phosphorylation of the insulin receptor.

Cultured human lymphocytes were exposed to RPMI-1640 media with or without insulin (10^{-7}M) for 10 minutes at 37°C. This is referred to as in vivo exposure. The incubation was stopped and the insulin receptors (IR) were partially purified on a wheat germ agglutinin column (Khan et al., 1986) as described in "Materials and Methods". In some experiments the receptors were further incubated in the presence and absence of insulin (10^{-7} M) for 60 minutes. This is referred to as in vitro exposure. The samples were then subjected to the autophosphorylation reaction (Roth et al., 1983) for 60 minutes at room temperature. They were then specifically immunoprecipitated with anti-human insulin receptor antibody (HIR) (Khan et al., 1989) as described in the "Materials and Methods". Both in vitro (Fig.1.A.lane.2) or in vivo (Fig.1.A.lane.3) exposure to insulin stimulated autophosphorylation of the 95 kDa band corresponding to the insulin receptor β-subunit. This observation is similar to the results reported by Kasuga et al. (Kasuga et al., 1982). As a first approximation of the possible presence of both phosphotyrosine and phosphoserine/phosphothreonine, the gels were washed with KOH, to remove ^{32}P-phosphate from serine and threonine residues, according to the procedure of Cooper et al. (Cooper et al., 1981). Comparison of the intensity of bands in the corresponding lanes revealed that the KOH wash resulted in the removal of 10 to 35 % of radioactivity (fig.1.B). Quantitation of the corresponding bands suggested that

FIGURE 1A

FIGURE 1B

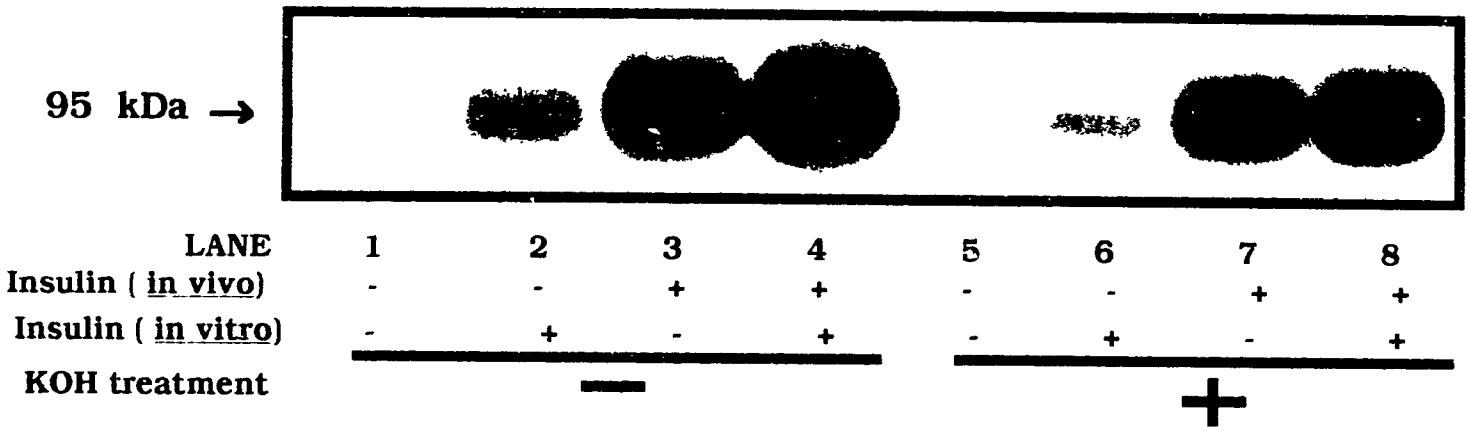


Fig.1.A&B. Autophosphorylation of partially purified insulin receptor stimulated with insulin. (A) Lymphocytes were treated with or without insulin in vivo (as described above). IR was then partial purified. Equal amounts of insulin receptor (IR) (390fmoles) were autophosphorylated at 22°C under these following conditions and lanes: Lane (1,5) IR without insulin in vivo or in vitro (10 minutes at 37°C), Lane (2,6) IR with insulin (10-7M) for 60 minutes in vitro at 4 C, Lane (3,7) IR with insulin (10-7M) for 10 minutes in vivo at 37°C or Lane (4,8) IR with insulin (10-7M) in vivo (10 minutes at 37°C) and in vitro (60 minutes at 4°C). Once IR's were autophosphorylated, the were immunopreciptated, resolved by SDS-PAGE and subjected to autoradiography as described under "Materials and Methods". (B) Gels from a parallel experiment were treated with KOH.

although tyrosine was the predominant amino acid labelled, a significant amount of phosphoserine and/or phosphothreonine was present (Fig1.C).

To confirm this observation, phosphoamino acid analysis was performed on the beta subunit of the phosphorylated receptor exposed to insulin in vitro and in vivo. The 95 kDa band shown in Fig.1.A,lane4 was excised from the gel and the radiolabelled protein was extracted and hydrolyzed as described in "Materials and Methods". Phosphoamino acids were resolved by Thin Layer Electrophoresis (TLE). The phosphotyrosine and phosphoserine bands were quantified by laser densitometry and this revealed the presence of phosphotyrosine and phosphoserine in the relative amounts of 81% and 19% respectively (Fig.2). No phosphothreonine was detected in this or in any other subsequent phosphoamino acid analysis (PAAA).

3.2 In vivo activation time course

The observation that prior exposure of intact cells to insulin in vivo stimulated the in vitro assayed serine phosphorylation of the partially purified IR prompted us to examine the time course of this activation. Lymphocytes were exposed to 10^{-7}M insulin in vivo for 1 to 60 minutes. This exposure stimulated total phosphorylation. The maximum phosphorylation was observed at 1 minute. Removal of phosphoserine/phosphothreonine with KOH revealed a similar time course with a lesser degree of phosphorylation (Fig.3A,B,C). This suggests that insulin-stimulated receptor serine kinase activation

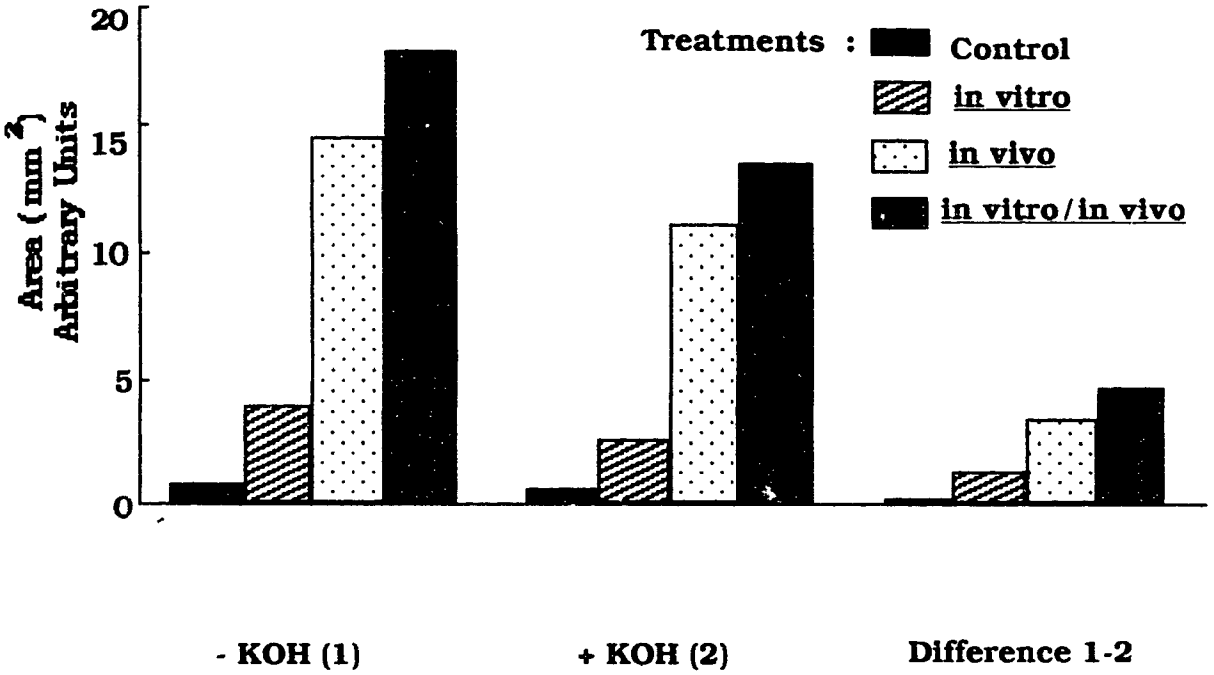


Fig.1.C. Quantitation of band intensities of partially purified insulin receptor after autophosphorylation and alkali treatment from Fig.1.A & B.

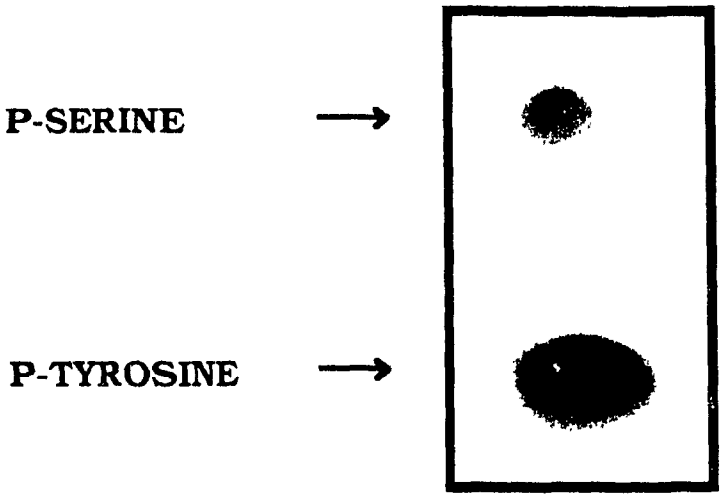
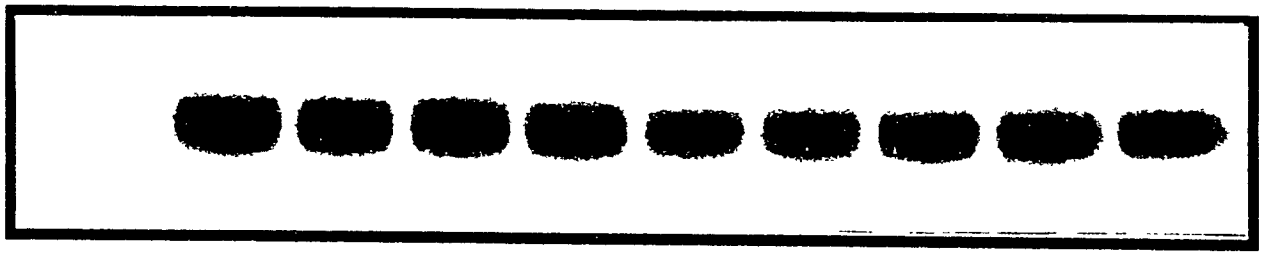


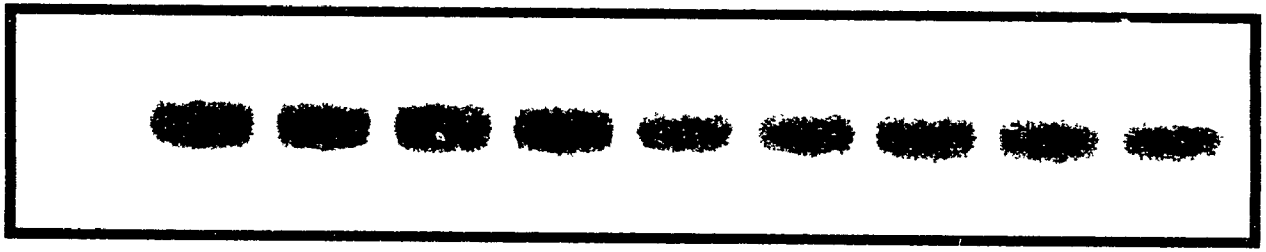
Fig.2. Phosphoamino acid analysis (PAAA) of partially purified insulin receptor stimulated with insulin in vitro and in vivo. IM-9 lymphocytes were stimulated with insulin (10-7M) in vivo (10 minutes at 37°C) . The IR was partially purified and was incubated with insulin (10-7 M) in vitro (60 minutes at 4°C). Equal amounts of IR were autophosphorylated, immunoprecipitated, resolved by SDS-PAGE and subjected to autoradiography as described under "Materials and Methods". From Fig.1. A lane.4, the 95-kDa band from the gel was excised and the radiolabeled protein was extracted and hydrolyzed as described in " Materials and Methods". Phosphoamino acids were resolved by thin-layer-electrophoresis (TLE). The positions of phosphotyrosine (P-Tyrosine) and phosphoserine (P-Serine) markers are noted (arrows). The P-Tyrosine and P-Serine spots were quantified by a LKB ultrascan XL enhanced laser densitometer with two dimensional analysis.

FIGURE 3A



Incubation Time (minutes) 0 1 2 5 10 15 20 30 45 60

FIGURE 3B



Incubation Time (minutes) 0 1 2 5 10 15 20 30 45 60

Fig.3.A&B. Time course of insulin receptor phosphorylation stimulated with insulin in vivo. (A) Intact IM-9 lymphocytes were treated with 10-7M insulin for 0 , 1 , 2 , 5 , 10 , 15 , 20 , 30 , 45 , 60 minutes. The treatments were terminated by addition of ice cold "stopping buffer" containing protease and phosphatase inhibitors and rapid freezing to -70°C. The cells were thawed, homogenized and solubilized and the insulin receptors partially purified as described. The IR's were then autophosphorylated at 22°C in vitro, immunoprecipitated, resolved by SDS-PAGE, and subjected to autoradiography. (B) Gels from a parallel experiment were treated with KOH.

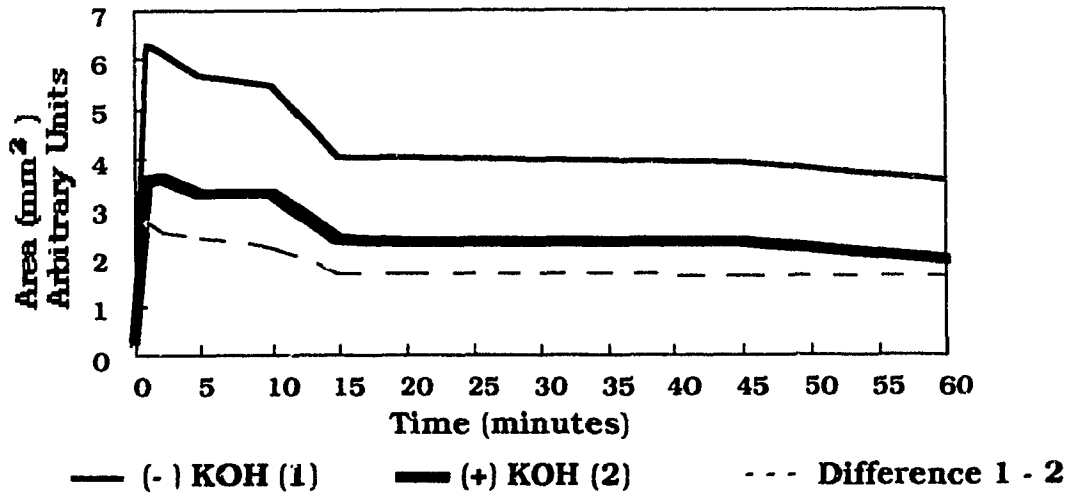


Fig.3.C. Graphical representation of the time course of phosphorylation of insulin receptor stimulated in vivo from Fig.3.A & B.

followed a similar time course. To confirm these results the experiment was repeated at specific times with PAAA (Fig4.A,B,C,D).

From the assessment of the relative amounts of ^{32}P-phosphotyrosine from the PAAA and the total ^{32}P-labelling of the receptor beta subunit, tyrosine autophosphorylation peaked at 1 minute followed by a decrease by 10 to 15 minutes and then remained relatively constant. Similarly, receptor serine phosphorylation also peaked at 1 minute and gradually diminished (Fig.4.D). From 1 to 60 minutes exposure, the proportion of serine phosphorylation remained constant (Fig.3C).

The decrease in tyrosine autophosphorylation with time of exposure of intact cells to insulin may have been due to tyrosine phosphorylation that had taken place in vivo and the subsequent decrease in the availability of sites for phosphorylation. Alternatively, the decrease may correlate with decreased insulin receptor enzyme activity which has been well documented to depend on the extent of tyrosine phosphorylation of the β-subunit, particularly in the regulatory region (Tyr-1146, 1150, and 1151) (Herrera et al., 1986).

As one method to distinguish these possibilities, we isolated the IR as described after various times (0-60 minutes) of exposure of intact cells to insulin. Equal amounts of partially purified receptors were separated by SDS-PAGE, transferred to nitrocellulose membranes and phosphotyrosine content of the beta subunit was quantified by Western blotting with anti-phosphotyrosine antibodies.

FIGURE 4A

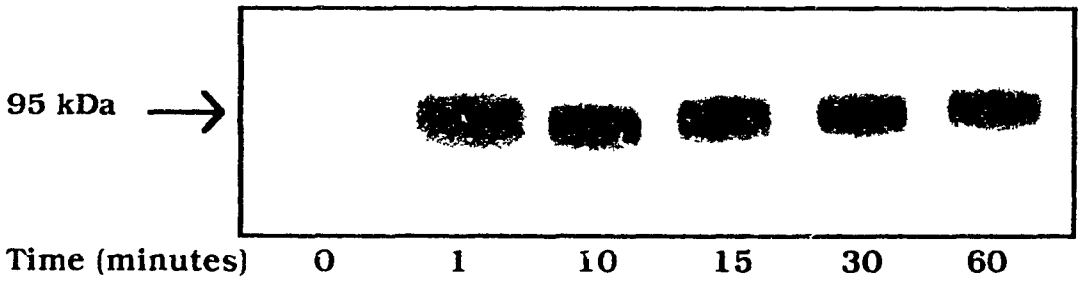


FIGURE 4B

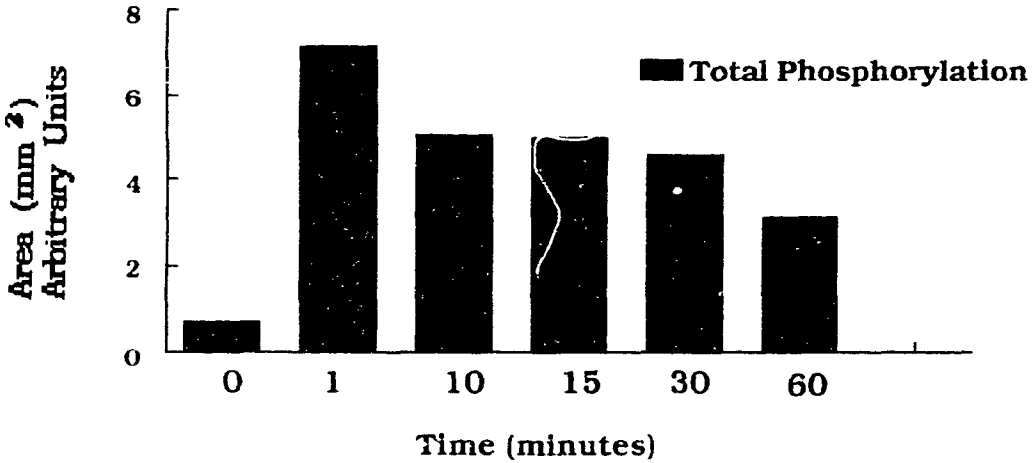


Fig.4.A&B. Time course of total autophosphorylation of partially purified insulin receptor stimulated in vivo with insulin. (A) IM-9 lymphocytes were stimulated in vivo with 10-7M insulin for 0 , 1 , 10 , 15 , 30 and 60 minutes. The IR was partially purified and equal amounts of partially purifiedIR were autophosphorylated at 22°C, immunoprecipitated, resolved on SDS-PAGE and subjected to autoradiography as described under "Materials and Methods". (B) Autophosphorylation band intensities from autoradiographs (Fig.4.A) were quantified by densitometry.

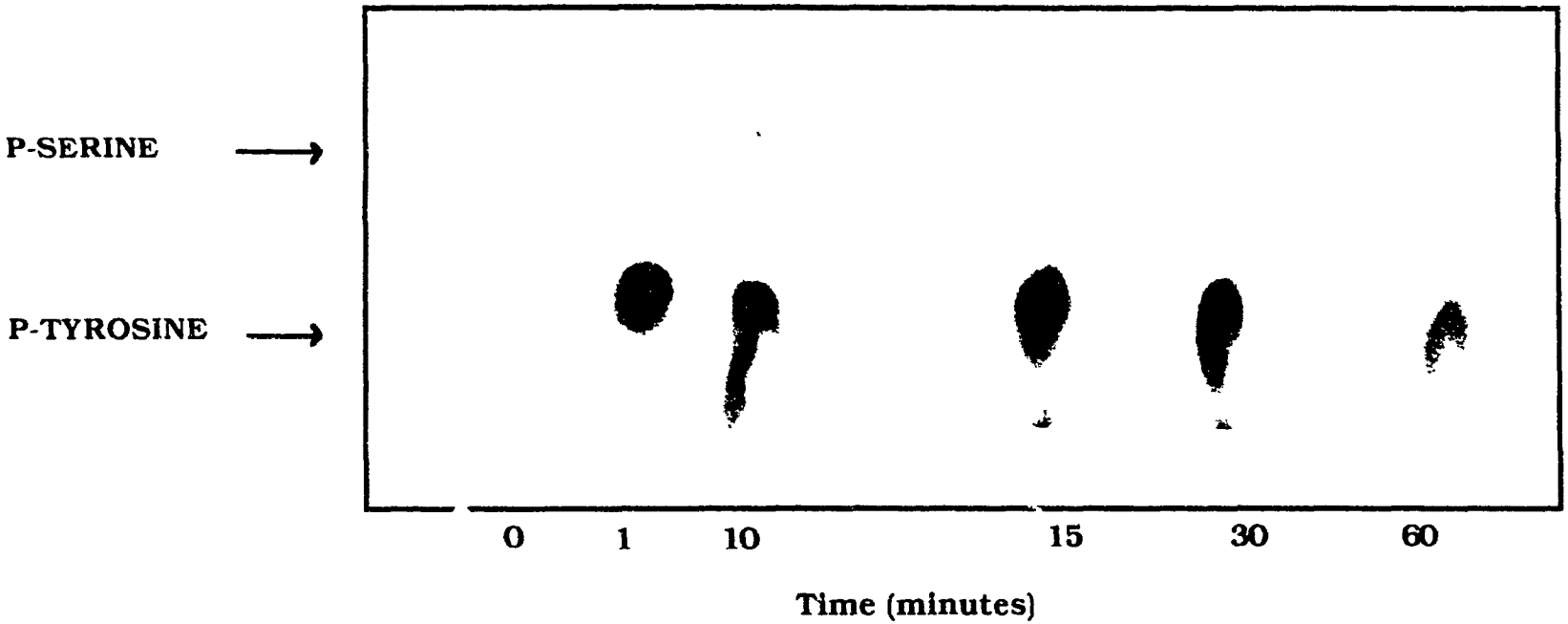


Fig.4.C. Phosphoamino acid analysis (PAAA) of partially purified insulin receptor stimulated in vivo with insulin for the indicated times. The 95-kDa band shown in Fig.4.A was excised from the gel, and the radiolabelled protein extracted and hydrolyzed as described under "Materials and Methods". Phosphoamino acids were resolved by Thin Layer Electrophoresis (TLE). P-Tyrosine and P-Serine markers are noted (arrows). The P-Tyrosine and P-Serine positions were quantified by a LKB ultrascan XL enhanced laser densitometer with two dimensional analysis.

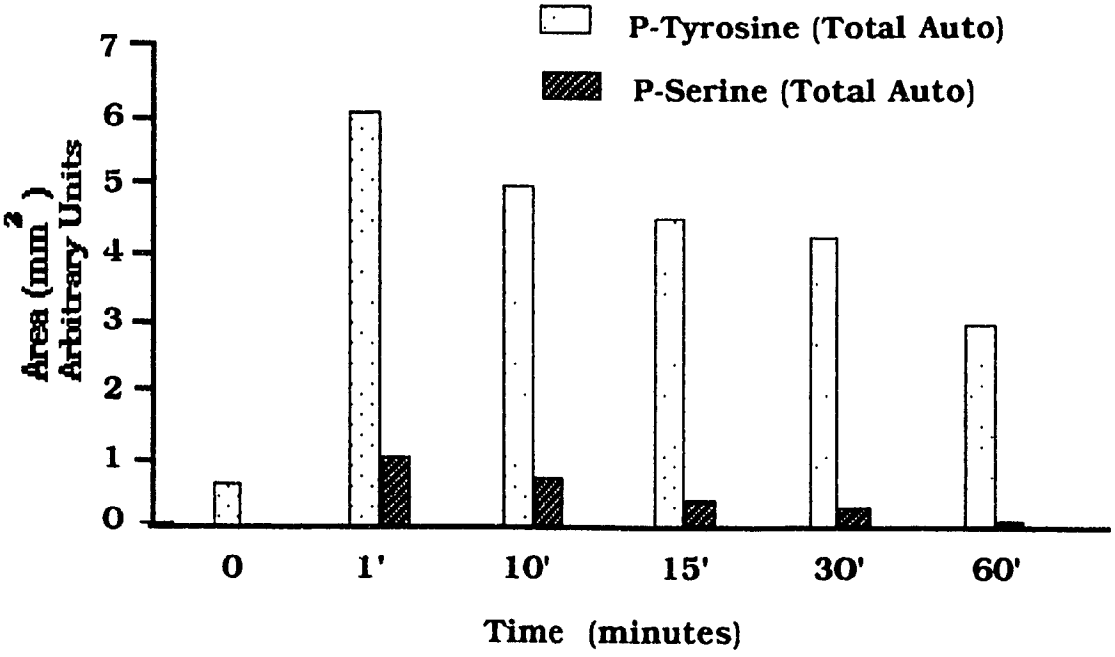


Fig.4.D. The content of 32P-tyrosine and 32P-serine in partially purified insulin receptor stimulated in vivo with insulin as determined from PAAA (Fig.4.C). Total 32P-Tyrosine and 32P-Serine incorporation was determined by the relative amounts from PAAA and the total 32Phosphate incorporation quantified by scanning the 95 kDa band representing the beta subunit as described in the "Materials and Methods".

Peak in vivo tyrosine phosphorylation was evident at 1 minute and remained elevated through 15 minutes of exposure to insulin (Fig.5.A&B). This was followed by a decrease after 30 minutes and a return to near basal levels by 60 minutes. These data strongly suggest that at least part of the decrease in in vitro measured autophosphorylation observed after longer times of in vivo exposure is due to decreased receptor enzyme activity associated with a dephosphorylation of tyrosine residues. We noted above that receptor serine kinase activity followed a similar time course to tyrosine phosphorylation. This may also be secondary to occupation of serine phosphorylation sites on the beta subunit or an actual decrease in the serine kinase activity. The latter possibility was considered more likely as the activation of this "serine kinase" by insulin both in vitro and in vivo suggested that tyrosine phosphorylation of the receptor may be required for serine kinase activation.

3. 3 In vitro time course of tyrosine and serine phosphorylation.

To determine whether tyrosine and serine phosphorylation occur in an ordered fashion, partially purified control receptor and receptor that had been pre-exposed in vivo to insulin for one minute (pre-activated receptor) were incubated for various times (0-20 minutes) in vitro in the autophosphorylation reaction. The control receptor was pre-incubated with insulin (10^{-7}M) for 60 minutes at 4C in vitro. The control receptor, activated only in vitro, showed a rapid tyrosine autophosphorylation evident by 1 minute and reaching peak

FIGURE 5A

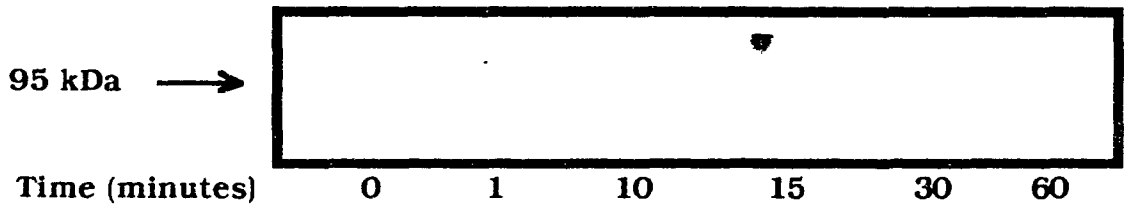


FIGURE 5B

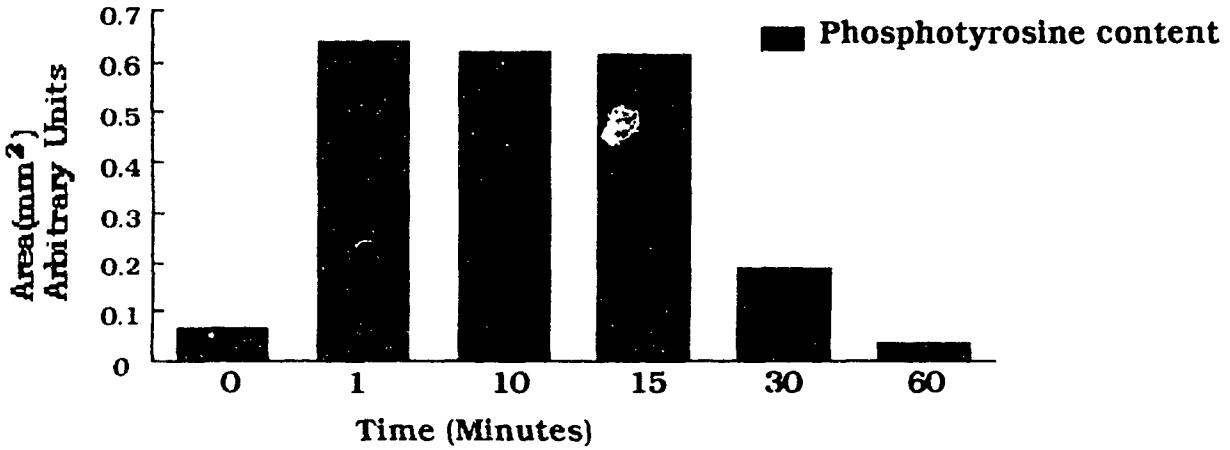


Fig.5.A&B. Western blots of partially purified insulin receptors stimulated with insulin in vivo. (A) IM-9 lymphocytes were stimulated in vivo with 10-7M insulin for 0 , 1 , 10 , 15 , 30 and 60 minutes. Partial purification of IR followed and resolved on SDS-PAGE, and transferred to a nitrocellulose membrane overnight. The nitro-cellulose was blocked and incubated with a rabbit anti-phospho-tyrosine antibody. The nitrocellulose membrane was washed and incubated with radiolabelled goat anti-rabbit antibody and washed again. The nitrocellulose membrane was then dried and subjected to autoradiography as described under "Materials and Methods". (B) Western blot band intensities from autoradiographs (Fig.5.A) were quantified by densitometry.

incorporation by 20 minutes (Fig.6A-E). Incubation for 60 minutes did not reveal significantly greater incorporation of ^{32}P-phosphotyrosine (data not shown). However, serine phosphorylation was not detectable until 5 minutes of autophosphorylation and increased up to 20 minutes (Fig.6 C-E). In contrast, the pre-activated receptor demonstrated rapid beta subunit tyrosine and serine phosphorylation evident at 1 minute and both reached maximum incorporation by 5 minutes (Fig.6C-E). These data suggest that receptor tyrosine phosphorylation precedes and is required for serine phosphorylation. It may be noted that total autophosphorylation was lowest after 1 and 3 minutes of phosphorylation of the in vitro activated receptor so that the absence of ^{32}P-serine, may have been due to an inability to detect it. However, longer exposure of the ^{32}P-phosphoamino acids (phosphotyosine and phosphoserine bands) showed that even allowing intense ^{32}P-tyrosine labelling did not reveal significant ^{32}P-serine.

3.4 Characterization of insulin receptor serine kinase .

Serine phosphorylation of the IR has been previously detected in vivo and in vitro. This phosphorylation has been observed in vivo in the basal state (Ballotti et al., 1987) as well as after stimulation by PMA (Phorbol-12-Myristate-13-acetate), a diacylglycerol analogue which activates the serine /threonine kinase, protein kinase C (Takayama et al. 1984, 1988), and after exposure to insulin (Kasuga et al., 1982). The receptor serine phosphorylation stimulated by insulin has been postulated to be catalyzed by a putative insulin receptor serine kinase (IRSK) (Lewis et al., 1990). A previous report suggested that the IRSK

Figure 6A

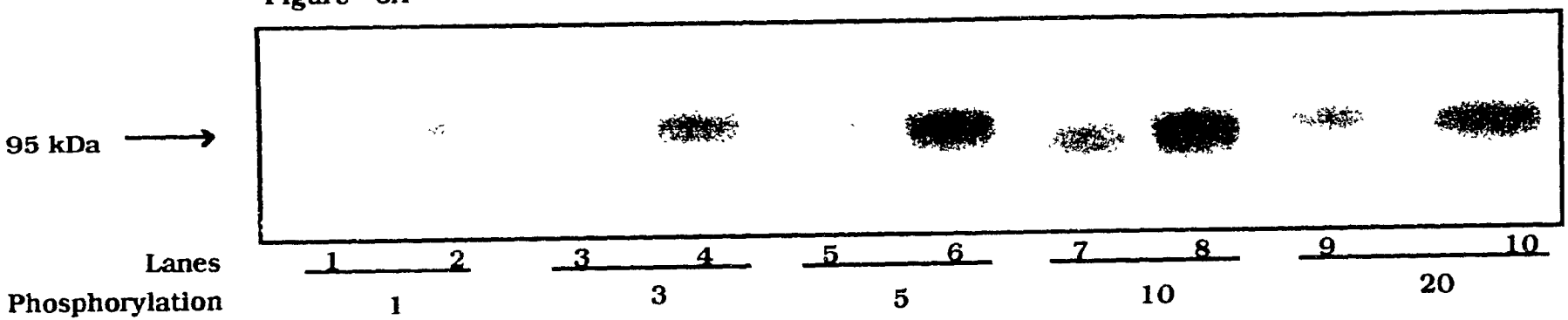


Figure 6B

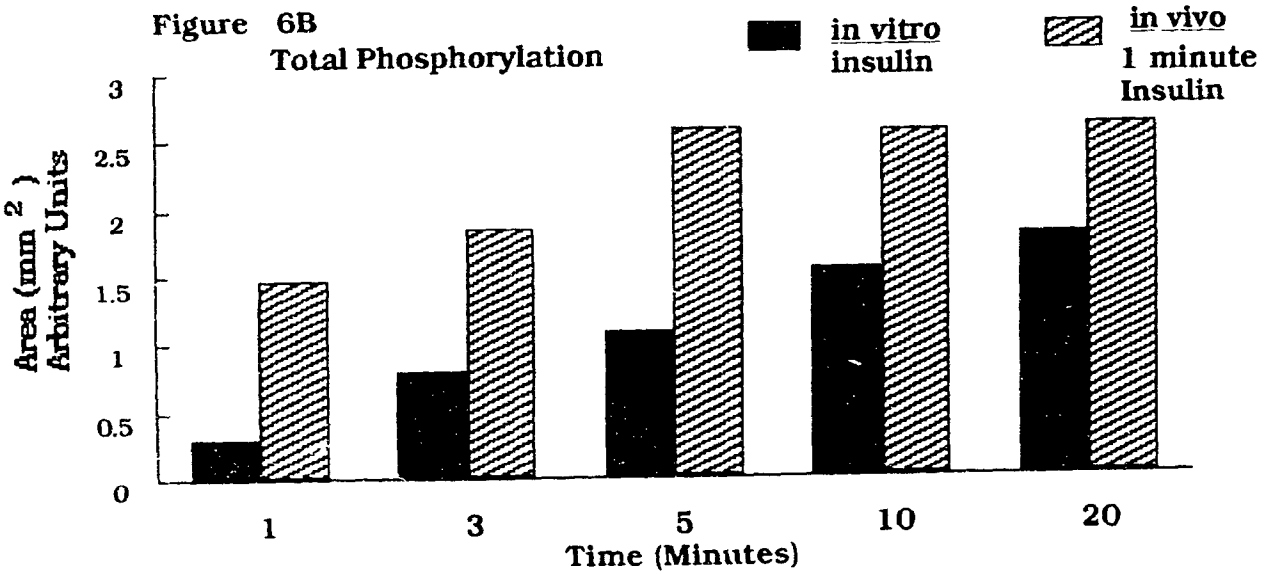


Fig.6.A&B. Time course of the autophosphorylation reaction of partially purified insulin receptors stimulated with insulin in vivo or in vitro. (A) IM-9 lymphocytes were (even numbered lanes) or were not treated in vivo for 1 minute with 10-7 M insulin at 37 C. Partial purification of the IR followed and untreated IR were then treated in vitro with 10-7M insulin for 60 minutes at 4 C (odd numbered lanes). Equal amounts of partially purified IR were autophosphorylated for 1(lanes1&2), 3(lanes3&4), 5 (lanes5&6), 10(lanes7&8), 20 (lanes9&10) minutes at 22 C. IR's were immunoprecipitated, resolved by SDS-PAGE and subjected to autoradiography as described under "Materials and Methods". (B) Intensities of the 95 kDa bands from autoradiographs (Fig.6.A) were quantified by densitometry.

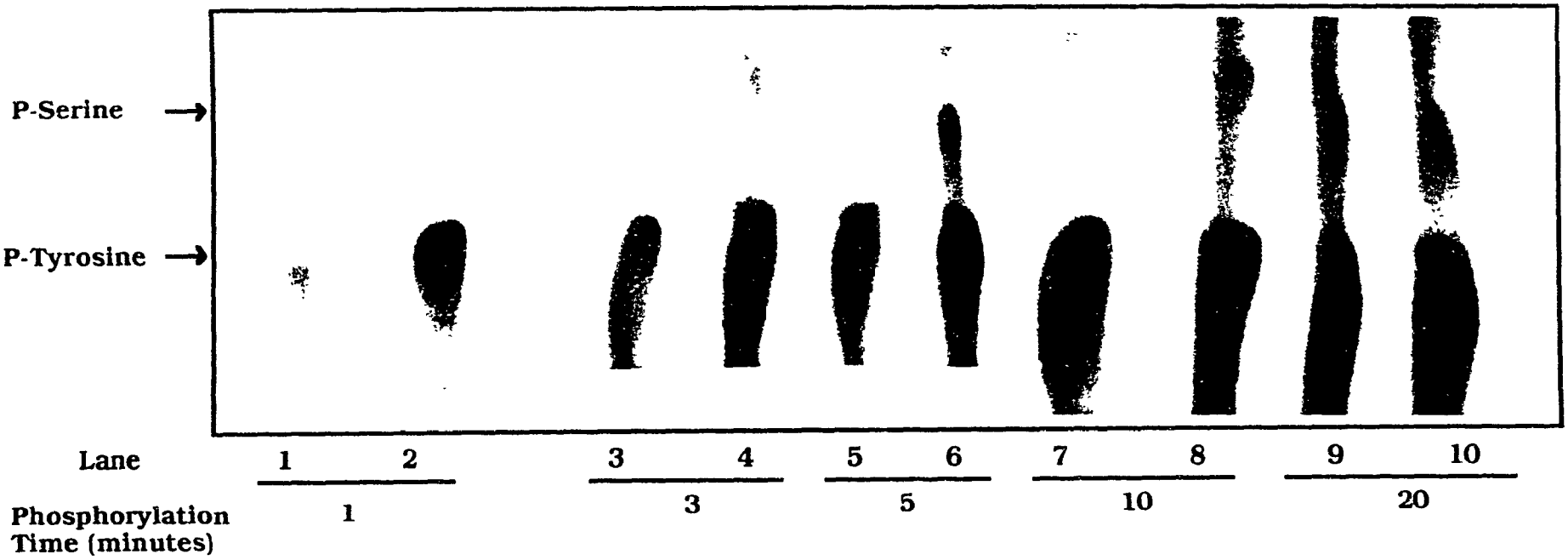


Fig.6.C. PAAA of the time course of the autophosphorylation reaction of partially purified IR stimulated with insulin in vivo or in vitro. The 95 kDa-bands shown in Fig.6.A were excised from the gel, the radiolabelled protein extracted and hydrolyzed as described under "Materials and Methods". Phosphoamino acids were resolved by Thin Layer Electrophoresis (TLE). P-Tyrosine and P-Serine are noted (arrows). The P-Tyrosine and P-Serine positions were quantified by a LKB ultrascan XL enhanced laser densitometer with two dimensional analysis.

Figure 6D

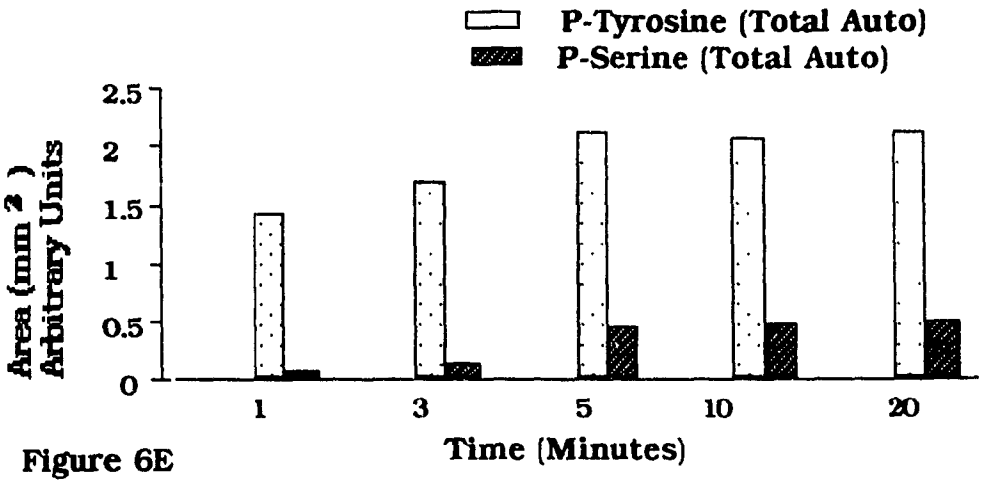


Figure 6E

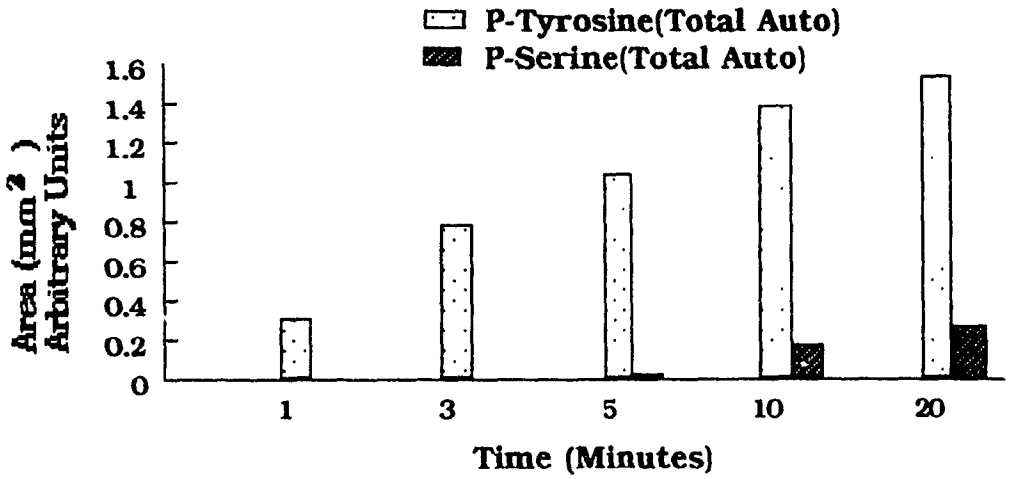


Fig.6.D&E. Quantification of 32P-Tyrosine and 32P-Serine content in the phosphorylation reaction time course of partially purified IR stimulated with insulin in vivo (upper panel D) or in vitro (lower panel E).

may be separated from the IR by washing the receptors bound to the WGA column with 1M NaCl thereby eluting only the IRSK (Sale et al.. 1988).

To determine whether the IRSK activity in our preparation could be removed , the WGA column was washed with 1M NaCl prior to elution of the IR as described in "Materials and Methods". Although total phosphorylation was decreased in the 1M NaCl washed receptor which was insulin activated either in vitro or in vivo (Fig.7.A&B), the extent of serine phosphorylation was not altered (Fig.7C&D). Thus. there was no apparent removal of the insulin stimulated IRSK activity by high salt wash.

It has been suggested that with stimulation of protein kinase C (Bollag et al. 1986) or cAMP dependent, protein kinase A (Roth et al., 1987), the serine phosphorylation of the IR beta subunit may be catalyzed by a putative IRSK which may be activated by these other serine kinases. To determine whether such an IRSK activity was observable in our preparation the intact lymphocytes were exposed to 5nM and 100nM PMA for 30 minutes prior to isolation and partial purification of the receptor. These treatments did not result in any increased serine kinase activity associated with the receptor preparation (Fig.8A-D). Similar to previous reports (Takayama et al., 1984; Takayama et al., 1988; Haring et al., 1986) the extent of total phosphorylation (Fig.8A&B) and tyrosine autophosphorylation (Fig.8C&D) stimulated by insulin in vivo was decreased by prior exposure of cells to PMA.

Figure 7A

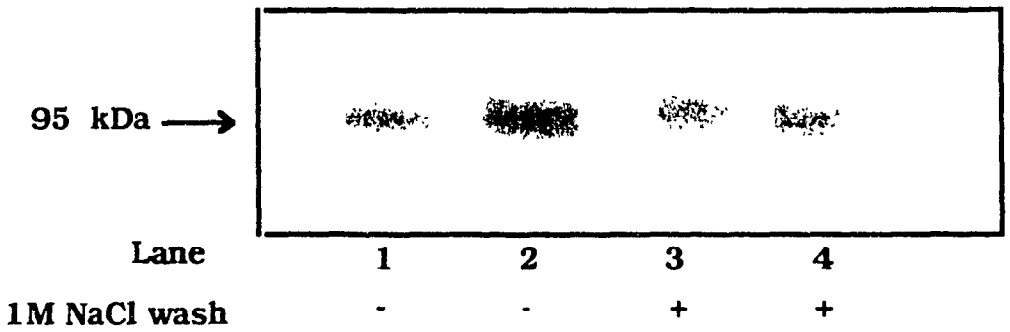


Figure 7B

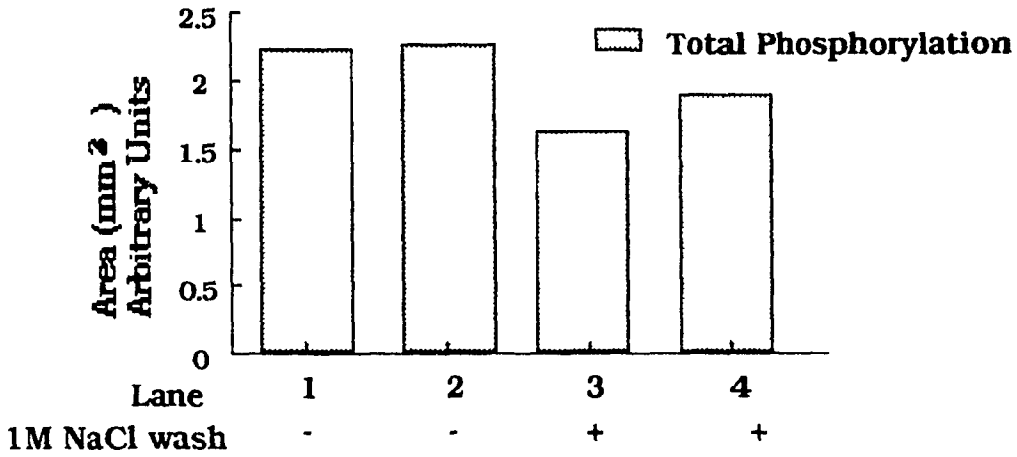


Fig.7.A&B. Total autophosphorylation of partially purified IR stimulated with insulin in vivo or in vitro and washed with 1 M NaCl. (A) IM-9 lymphocytes were (lane 2,4) or were not (lane 1,3) stimulated with 10-7M insulin in vivo for 1 minute at 37°C and washed with 1M NaCl (lane 3,4). Partial purification of the IR followed and unstimulated IR were stimulated with 10-7M insulin in vitro for 60 minutes at 4°C (lane 1,3). Insulin receptors were autophosphorylated at 22C, immunoprecipited, resolved by SDS-PAGE and subjected to autoradiography as described under "Materials and Methods". (B) Autophosphorylation band intensities from autoradiographs (Fig.7.A) were quantified by densitometry.

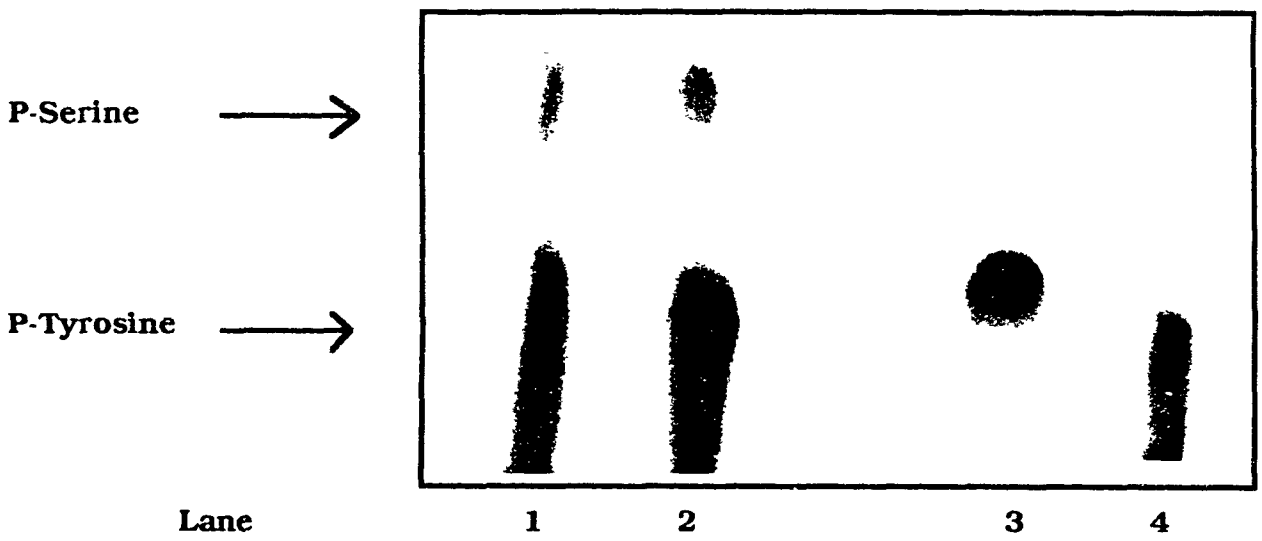


Fig.7.C. PAAA of partially purified, 1M NaCl washed IR which had been stimulated with insulin in vivo or in vitro. The 95-kDa band shown in Fig.7.A was excised from the gel and the radiolabelled protein was extracted and hydrolyzed as described under "Materials and Methods". Phosphoamino acids were resolved by Thin Layer Electrophoresis (TLE). The positions of P-Tyrosine and P-Serine are noted (arrows). The P-Tyrosine and P-Serine positions were quantified by a LKB ultrascan XL enhanced laser densitometer with two dimensional analysis and compared with standards.

Lane 1: in vitro insulin treatment of IR.
Lane 2: in vivo insulin treatment of IR.
Lane 3: in vitro insulin treatment of IR washed with 1M NaCl .
Lane 4: in vivo insulin treatment of IR washed with 1M NaCl .

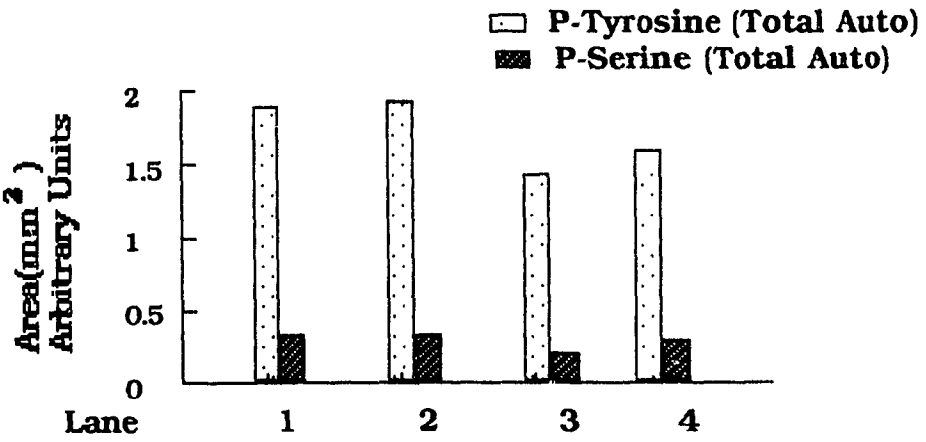


Fig.7.D. Quantification of P-Tyrosine and P-Serine content (for Fig.7.A) in 1M NaCl washed partially purified IR stimulated with insulin in vitro (lane 1. 3) or in vivo (lane 2, 4) and compared to the standard IR preparation in the same experiment.

Lane 1: in vitro insulin treatment of IR.
Lane 2: in vivo insulin treatment of IR.
Lane 3: in vitro insulin treatment of IR washed with 1M NaCl .
Lane 4: in vivo insulin treatment of IR washed with 1M NaCl .

Figure 8A

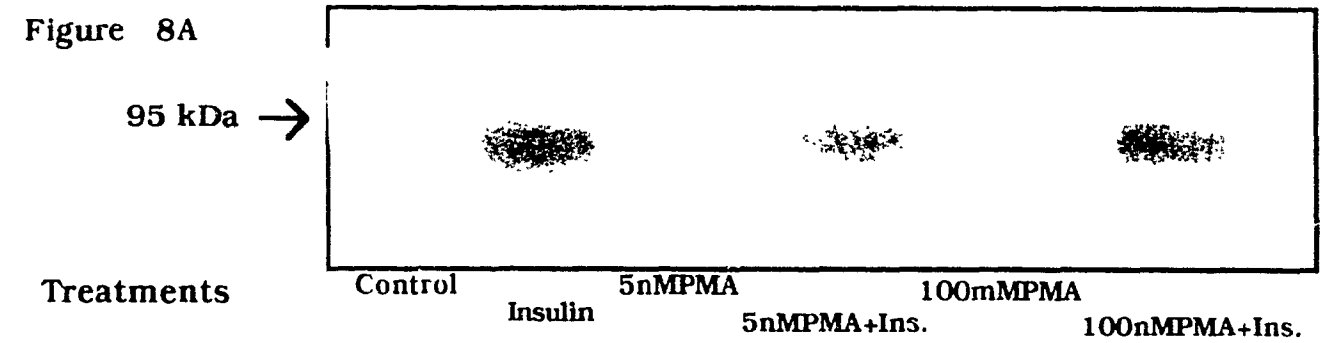


Figure 8B

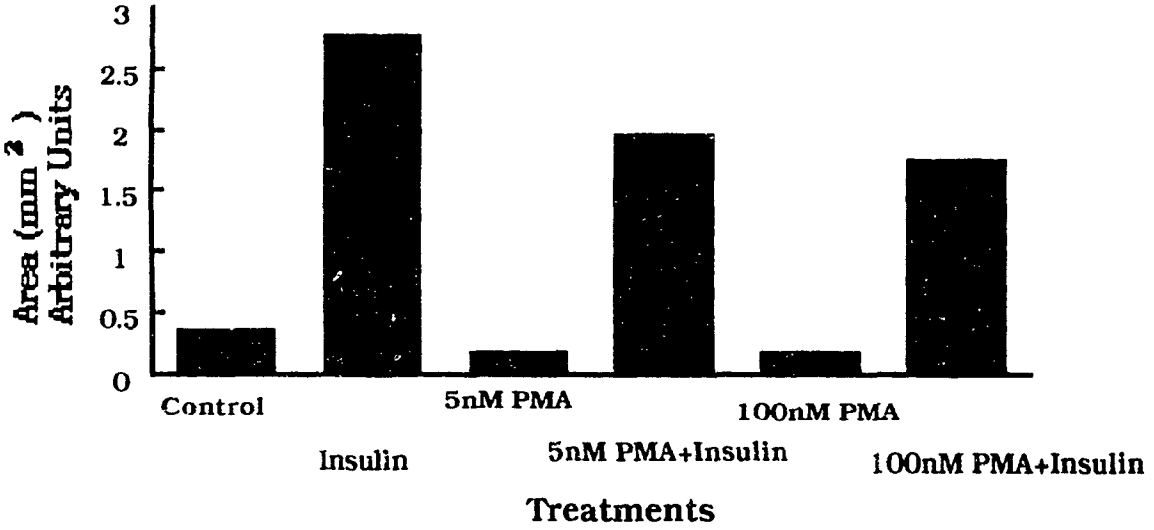


Fig.8.A&B. Effect of PMA on total phosphorylation of partially purified IR . (A) IM-9 lymphocytes were exposed or not to PMA (5 and 100 nM) for 30 minutes in vivo followed by treatment with or without insulin (10-7M) for 1 minute at 37°C. Insulin receptors were partially purified and allowed to undergo autophosphorylation for 60 minutes at 22°C as described in "Materials and Methods". The reaction was terminated and IR were immunoprecipitated, resolved by SDS-PAGE, and subjected to autoradiography . (B) Autophosphorylation band intensities from autoradiographs (Fig.8.A) were quantified by densitometry.

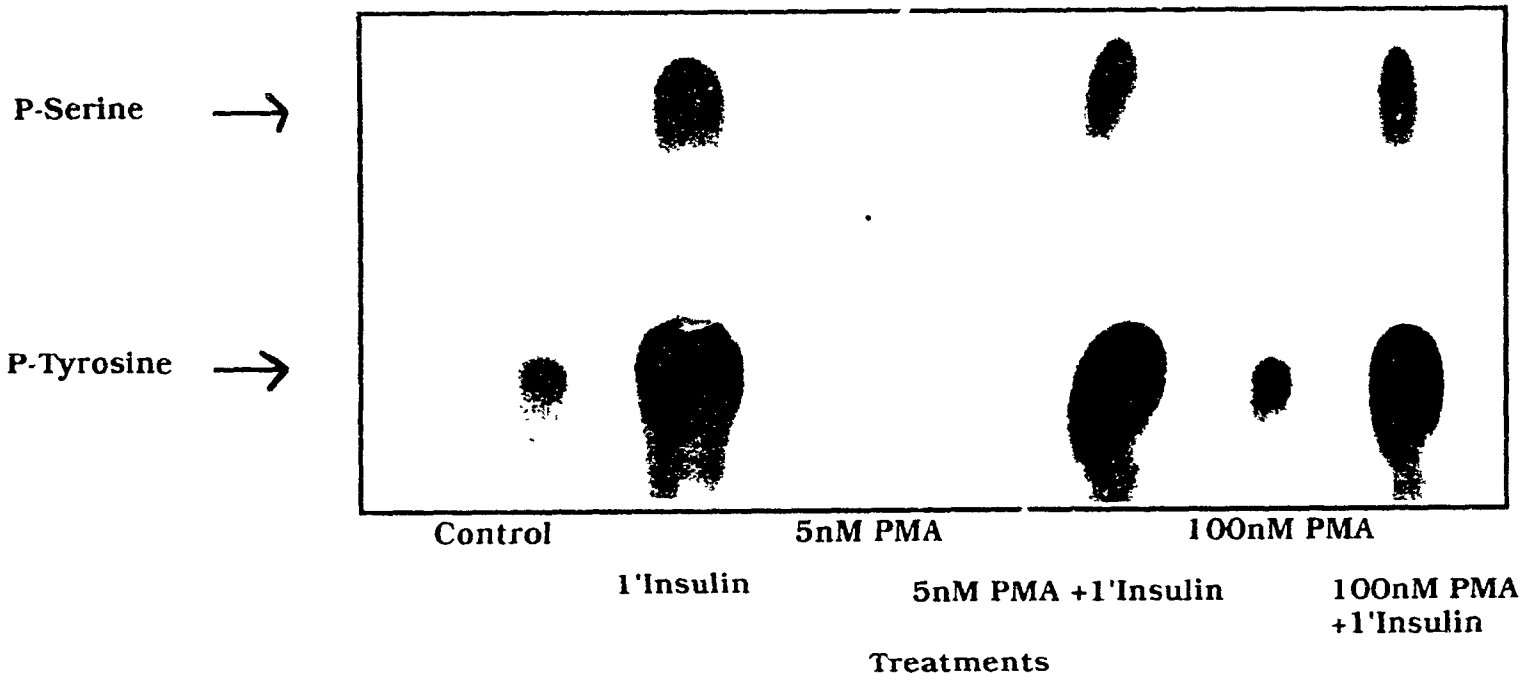


Fig.8.C. PAAA of partially purified IR treated with 5nM or 100 nM PMA in vivo. The 95-kDa band shown in Fig.8.A was excised from the gel and the radiolabelled protein was extracted and hydrolyzed as described under "Materials and Methods". Phosphoamino acids were resolved by Thin Layer Electrophoresis (TLE). The positions of P-Tyrosine and P-Serine are noted (arrows). The P-Tyrosine and P-Serine positions were quantified by a LKB ultrascan XL enhanced laser densitometer with two dimensional analysis.

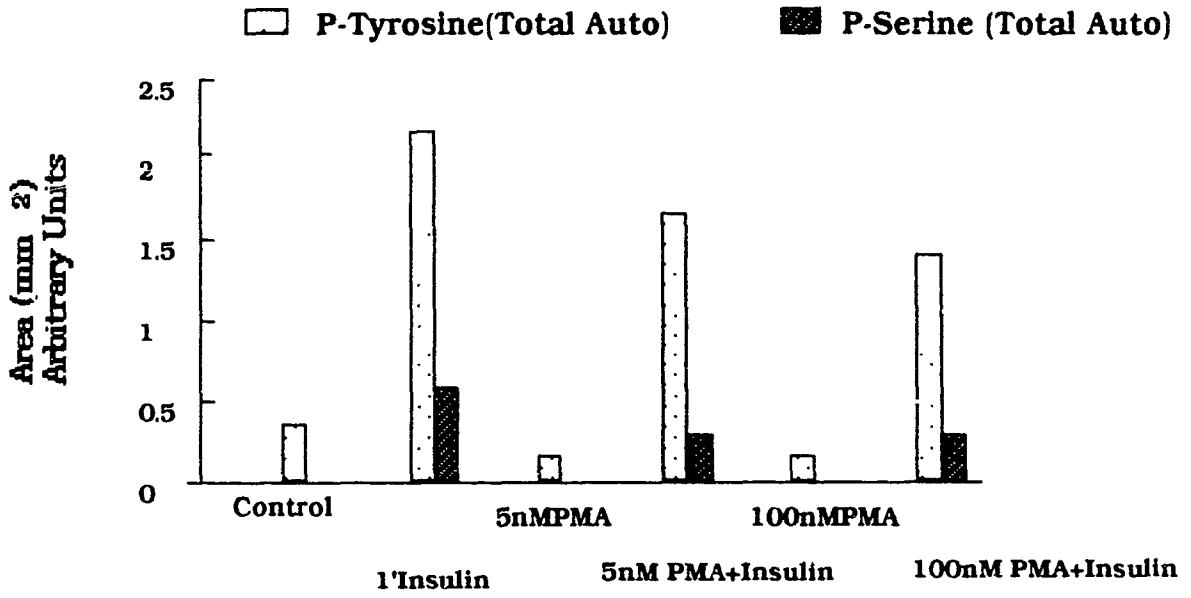


Fig.8.D. Quantification of P-Tyrosine and P-Serine content in autophosphorylated insulin receptor beta subunit from cells treated with 5nM or 100 nM PMA.

Interestingly, the proportion of serine phosphorylation stimulated by insulin in vivo remained the same in control and PMA pre-treated receptor (Fig.8C&D). Thus, PMA inhibited both tyrosine and serine phosphorylation to the same extent, again suggesting a close association of the two kinase activities. These data indicate that the insulin-stimulated serine kinase activity demonstrated in these preparations is unique and distinct from that associated with activation of protein kinase C.

The inhibition of in vivo insulin stimulated tyrosine phosphorylation implied by the decreased autophosphorylation activity assayed in vitro after PMA exposure was confirmed by anti-phosphotyrosine antibody western blots of receptors isolated from control and PMA pre-treated cells exposed to insulin for 1 minute (Fig.9A&B). As PMA concentration was increased , total and tyrosine phosphorylation decreased proportionally (Fig.10A-D).

3.5 Immunoprecipitation of autophosphorylated insulin receptor .

The close association of tyrosine and serine phosphorylation of the IR beta subunit under conditions of in vivo activation and inhibition suggested the possibility that serine kinase activity required prior receptor tyrosine phosphorylation as indicated by the time course of the in vitro insulin-stimulated phosphorylation. This may be secondary to activation of an IRSK or the ability of an IRSK to recognize the tyrosine phosphorylated receptor as a substrate or both.

Figure 9A

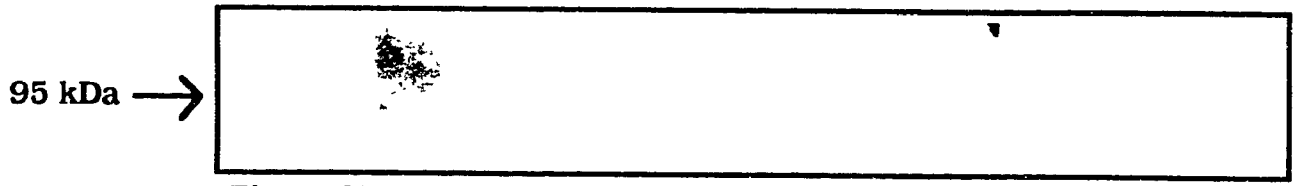


Figure 9B

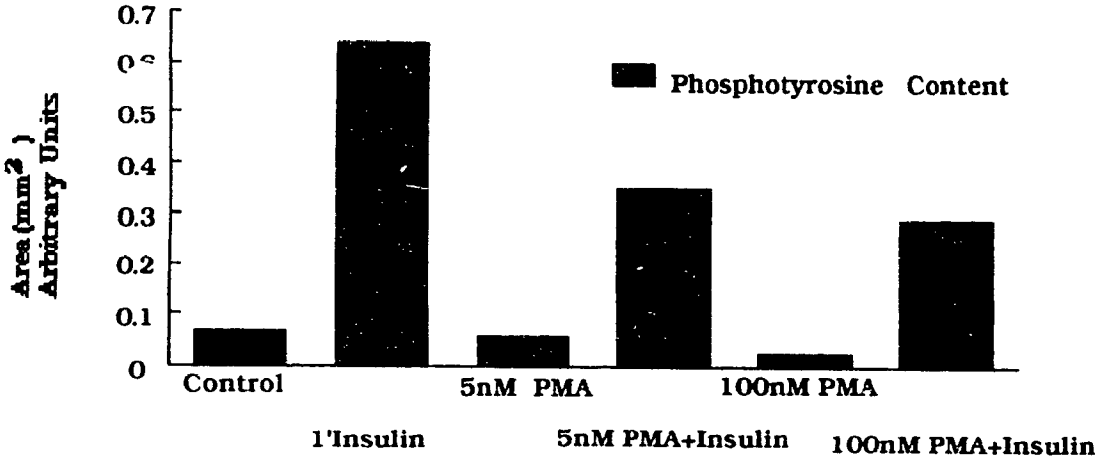


Fig.9.A&B. Anti- Phosphotyrosine antibody western blots of partially purified IR from IM-9 cells treated with 5nM or 100nM PMA in vivo. (A) IM-9 lymphocytes were treated in vivo with 5 or 100nM PMA for 30 minutes at 37°C and subsequently incubated with or without 10-7M insulin for 1 minute. Partial purification of the IR's followed and equal amounts of IR were resolved by SDS-PAGE and transferred to a nitrocellulose membrane overnight. This nitrocellulose membrane was blocked and incubated with a rabbit anti-phosphotyrosine antibody. The nitrocellulose membrane was washed and incubated a second time with radiolabelled goat anti-rabbit antibody and washed again. The nitrocellulose membrane was then dried and subjected to autoradiography as described under "Materials and Methods". (B) Western blot band intensities from radioautographs (Fig.9.A) were quantified by densitometry.

Figure 10 A

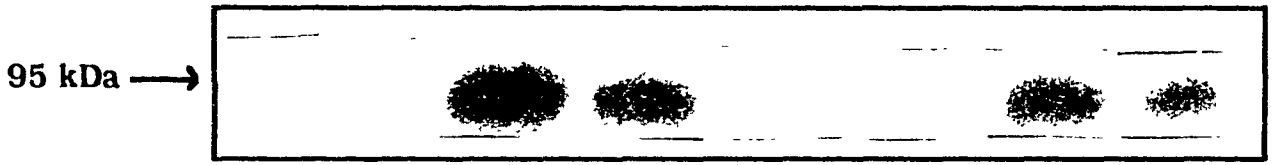


Figure 10 B

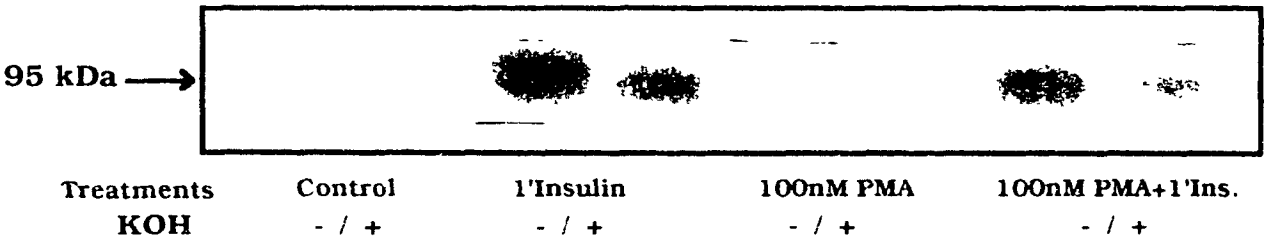


Fig.10.A&B. Effect of PMA exposure of lymphocytes on insulin receptor autophosphorylation. Cultured human lymphocytes were treated with (A) 5 or (B) 100nM PMA in vivo for 30 minutes at 37 C and incubated with or without 10-7M insulin for 1 minute after PMA treatment. The IR's were partially purified and then equal amounts of IR were allowed to undergo autophosphorylation. The IR's were immunoprecipitated, resolved by SDS-PAGE and subjected to radioautography as described under "Materials and Methods". Gels from a parallel experiment were alkali washed and subjected to autoradiography as described in "Materials and Methods".

Figure 10 C

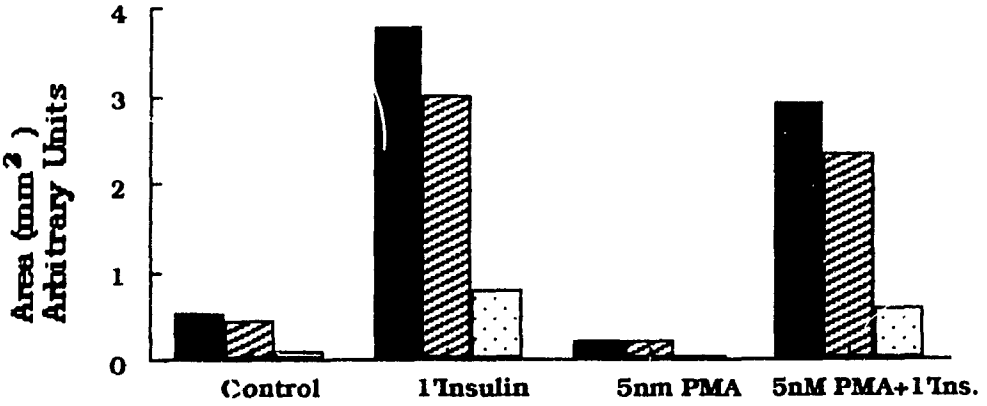


Figure 10 D

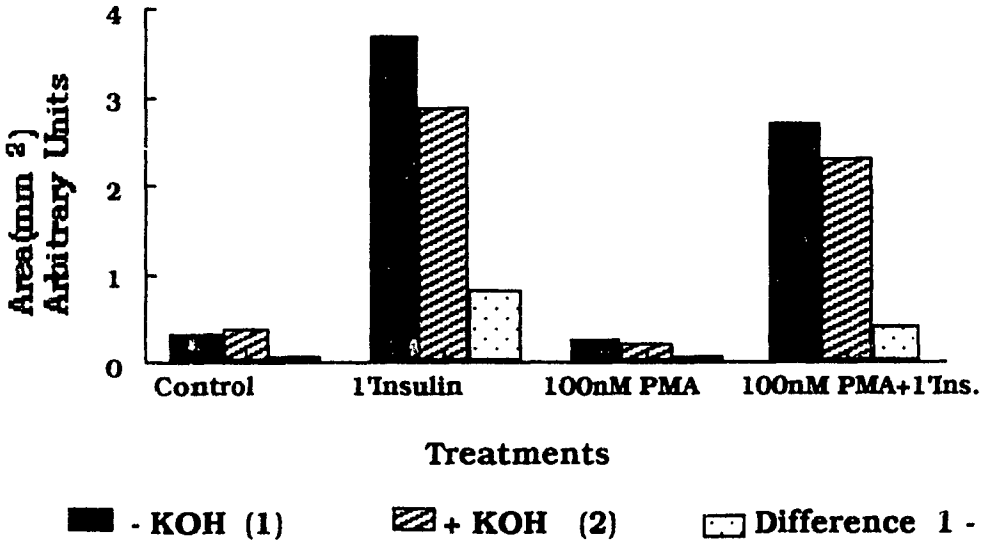


Fig.10.C&D. Effect of PMA exposure of lymphocytes on insulin receptor autophosphorylation. Quantitation of band intensities of partially purified insulin receptor after autophosphorylation and alkali treatment from Fig.10.A & B.

To determine whether insulin-stimulated serine phosphorylation occurs selectively on tyrosine phosphorylated receptors, the insulin receptors were immunoprecipitated with anti-phosphotyrosine antibodies after the autophosphorylation reaction. The receptors remaining in the supernatant (85% of total binding remained (data not shown) were subsequently immunoprecipitated with anti-human insulin receptor antibodies and separated by SDS-PAGE and subjected to autoradiography. Essentially all (>95%) of ^{32}P incorporated into the beta subunit of the in vivo activated receptor was immunoprecipitated with anti-phosphotyrosine or anti-human insulin receptor antibody (Fig.11A&B). Thus, only minimal or no radioactivity was detected in the receptor population immunoprecipitated with anti-human receptor antibody after prior immunoprecipitation with anti-phosphotyrosine antibody (Fig.11A&B). PAAA of the excised band revealed that similar amounts of phosphotyrosine and phosphoserine were immunoprecipitated with either anti-phosphotyrosine or anti-human insulin receptor antibodies (Fig.11C&D). These results are consistent with the concept that insulin stimulated serine phosphorylation occurs exclusively on the tyrosine phosphorylated receptors.

To confirm that all tyrosine and serine receptor phosphorylating activity was immunoprecipitated with anti-phosphotyrosine antibody, the IR preparation either in vitro activated or in vivo activated was allowed to undergo autophosphorylation as described, in the presence of unlabelled ATP. The tyrosine phosphorylated receptor population was then immunoprecipitated

Figure 11 A

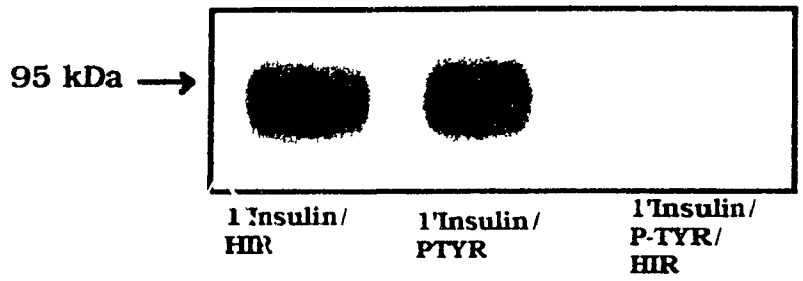
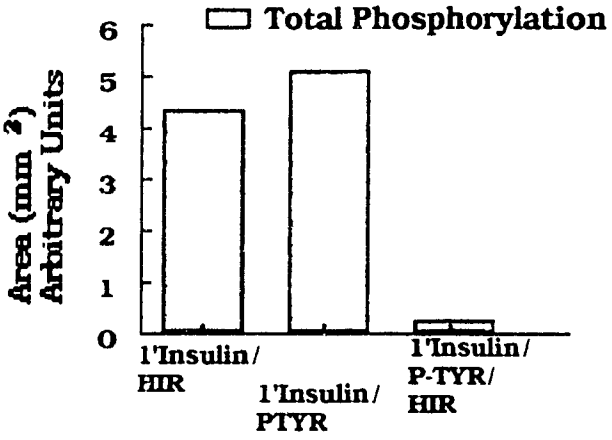


Figure 11 B



Treatments

(P-Tyr=Anti P-Tyr Antibody) (HIR=Human Insulin Receptor Antibody)

Fig.11.A&B. Immunoprecipitation of autophosphorylated insulin receptor with anti-phosphotyrosine antibody. (A) Lymphocytes were exposed to insulin (10-7M) in vivo for 1 minute at 37°C. The reaction was terminated and the insulin receptor partially purified as described. Equal amounts of IR were allowed to undergo autophosphorylation at 22°C for 60 minutes and immunoprecipitated with anti-human insulin receptor antibody (anti-HIR) as usual , anti-phosphotyrosine antibody or anti-phosphotyrosine antibody followed by HIR immunoprecipitation of the supernatant . Equivalent amounts and essentially all the radioactivity was immunoprecipitated with either anti-HIR or anti-phosphotyrosine antibody. (B) Autophosphorylation band intensities from autoradiographs (Fig.11.A) were quantified by densitometry.

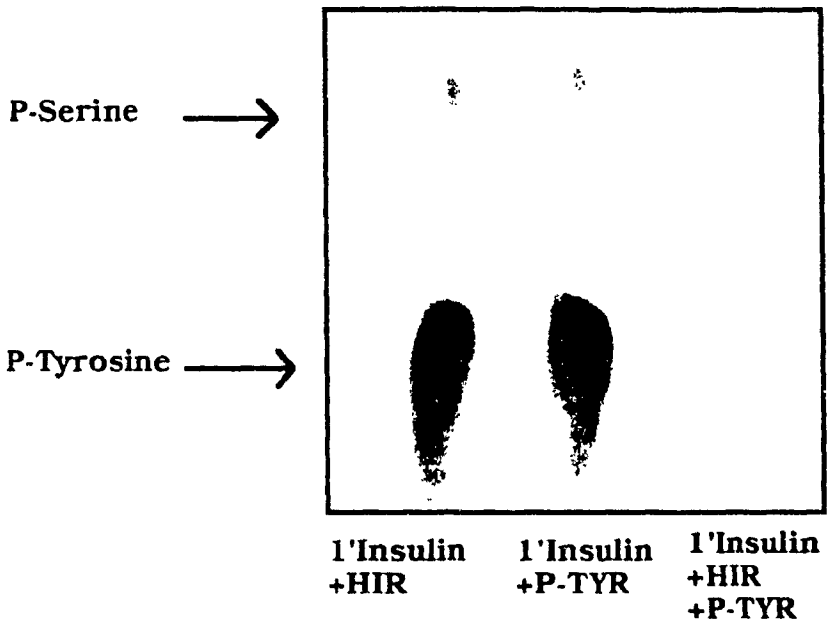


Fig.11.C. PAAA of partially purified IR stimulated with insulin in vivo and immunoprecipitated with anti-HIR antibody, anti-P-TYR antibody or both. The 95-kDa band shown in Fig.11.A was excised from the gel and the radiolabelled protein was extracted and hydrolyzed as described under "Materials and Methods". Phosphoamino acids were resolved by TLE and subjected to autoradiography as described in "Materials and Methods". The positions of P-Tyrosine and P-Serine positions are noted (arrows). The P-Tyrosine and P-Serine positions were quantified by a LKB enhanced XL laser densitometer with two dimensional anaylsis.

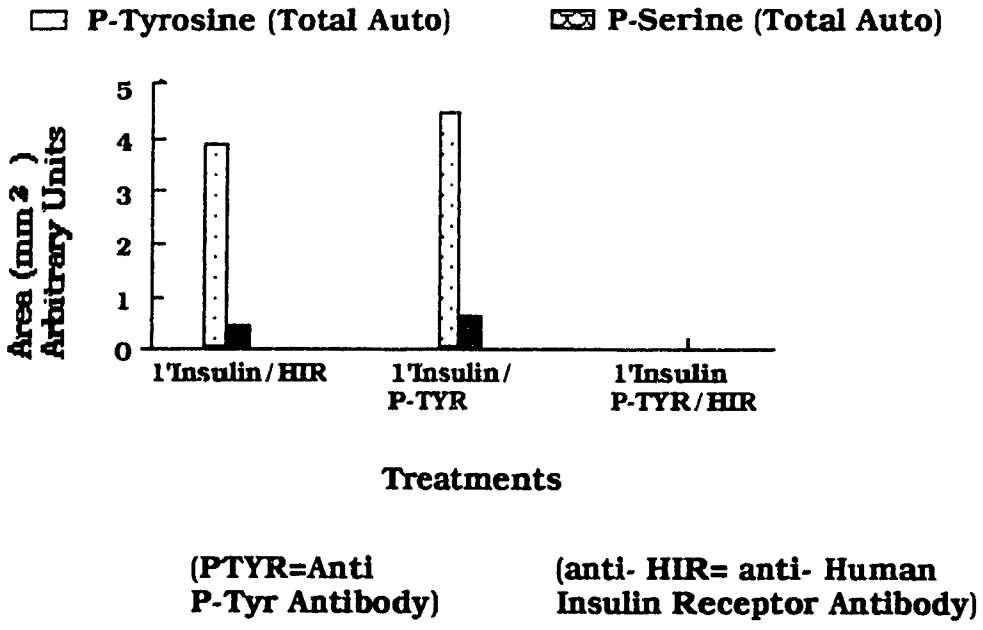


Fig.11.D. Quantification of total P-Tyrosine and P-Serine content in autophosphorylation of partially purified IR stimulated with insulin in vivo and immunoprecipitated with anti-HIR antibody, anti-P-TYR antibody or anti- HIR antibody after immunodepletion by anti-P-TYR. Equal amounts of 32P-tyr and 32P-ser were incorporated into the beta subunit that was immunoprecipitated by anti-HIR or anti-phosphotyrosine antibody indicating that essentially all the serine phosphorylation occured on the same receptor population as tyrosine phosphorylation.

with anti-phosphotyrosine antibodies and the receptors remaining in the supernatants were re-incubated in the presence of gamma ^{32}P-ATP. After 60 minutes, these receptors were immunoprecipitated with anti-human insulin receptor antibody and separated by SDS-PAGE. Autoradiographs revealed that no further labelling occurred even when insulin was re-added in vitro to the second incubation (Fig.12). These data indicate that there is a receptor population (85%) in the supernatant that cannot be activated, which does not undergo insulin stimulated tyrosine or serine phosphorylation.

3.6 Attempts to separate IRSK from the IR.

The above series of experiments suggested two possibilities: 1) that a distinct, tightly associated IRSK exists which is activated by receptor tyrosine phosphorylation and phosphorylates the receptor on serine residues or 2) the novel hypothesis that the insulin receptor serine kinase phosphorylation, as tyrosine phosphorylation, occurs via an autophosphorylation reaction.

As a first attempt to distinguish between these possibilities, we tried to separate the IRSK activity from the IR. Serine phosphorylation by a distinct enzyme should result in the physical separation of the enzyme from the substrate once the reaction has occured. Thus the insulin receptor preparation was pre-incubated with unlabelled ATP in an autophosphorylation reaction as described for 60 minutes at 22C. At the end of the reaction the phosphorylated IR was removed by immunoprecipitation with anti-HIR antibody and fresh, unstimulated

Figure 12 A

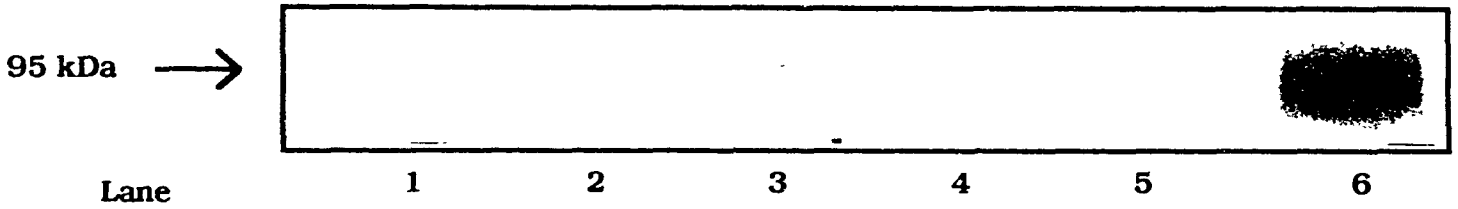


Figure 12 B

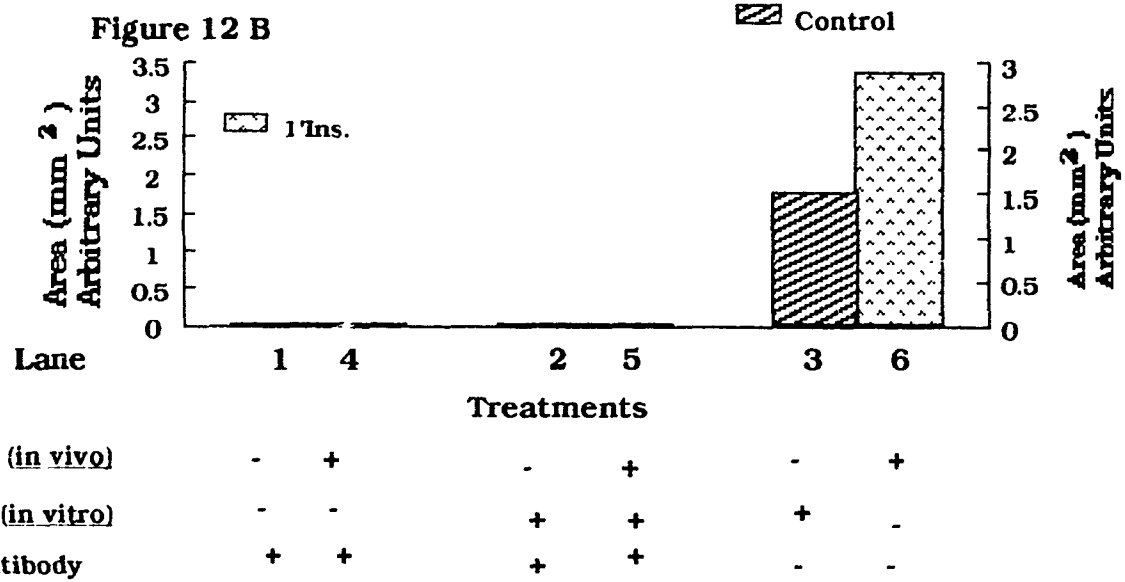


Fig.12. Autophosphorylation studies of partially purified IR stimulated with insulin in vivo or in vitro and immunoprecipitated with or without anti-P-TYR antibody. Lymphocytes were either exposed to insulin (10-7M) for 1 minute at 37°C in vivo (lane 4, 5, 6) or just incubated for 1 minute at 37°C (lane 1, 2, 3). IR's were subsequently partially purified as described in "Materials and Methods". Those not exposed in vivo were incubated with insulin (10-7M) for 60 minutes at 4°C in vitro (lane 1, 2, 3) . Once pre-exposed to insulin in vivo or in vitro, equal amounts of IR's were autophosphorylated with unlabelled 25uM ATP and immunoprecipitated with or without anti-P-TYR antibody overnight. The IR's remaining in the supernatants from unactivated or activated IR's in vivo were (lane 2,5) or were not (lane 1,4) incubated again with in vitro insulin (10-7M) and then were autophosphorylated in the presence of 25 μM γ- 32P-ATP , immunoprecipitated with anti-HIR, resolved by SDS-PAGE and subjected to autoradiography as described under "Materials and Methods". Insulin binding studies on supernatants were performed to determine recovery rate of pre-phosphorylated IR as described in "Materials and Methods". Lymphocytes treated with insulin in vivo (lane6) whose IR's were partially purified and partially purified IR' s treated with insulin in vitro (lane 3) were autophosphorylated with γ- 32P - ATP after immunoprecipitation with control IgG and processed as above for comparison to lanes 1,2,4,5. The IR remaining in the supernatants were inactive and could not be stimulated by insulin to undergo autophosphorylation (in vitro insulin stimulated receptor, lane 1&2) (in vivo insulin stimulated IR , lane4&5).

IR added to the supernatant and a second autophosphorylation allowed to proceed in the presence of γ ^{32}P-labelled ATP. When the first autophosphorylation was carried out with either in vitro or in vivo activated IR, no remaining IRSK activity could be detected in the supernatants with fresh control receptor (Fig.13A-D).

3.7 Inhibition of receptor tyrosine kinase activity .

Genistein is a compound which inhibits tyrosine autophosphorylation and tyrosine kinase activity relatively selectively by interfering with ATP binding (Akiyama et al., 1987). At a concentration of 5000 μM, genistein significantly inhibited in vitro insulin-stimulated autophosphorylation carried out for 60 minutes at 22°C (Fig.14A-C). When the gels from these experiments were washed with KOH a similar proportion of radioactivity was removed as in the absence of genistein. These results suggested that there was no preferential loss of phosphotyrosine over phosphoserine and no preferential inhibition of tyrosine kinase over serine kinase activity. (Fig.14A-C).

To test this further, both in vitro and in vivo insulin-stimulated receptors were allowed to undergo autophosphorylation for 60 minutes at 22°C in the presence and absence of 2000μM genistein which again significantly inhibited total receptor phosphorylation (Fig.15A&B). PAAA revealed that the proportion of ^{32}P-tyrosine and ^{32}P-serine were identical in the presence and absence of genistein

Figure 13 A

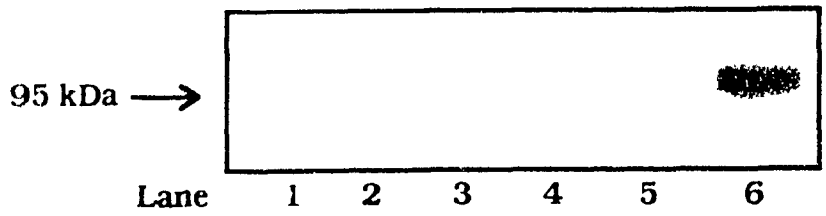


Figure 13B

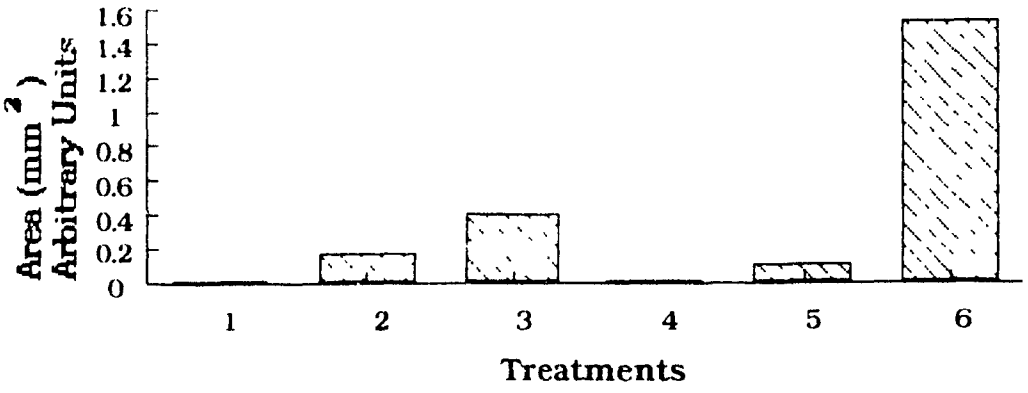


Fig.13.A&B. Autophosphorylation studies of unstimulated partially purified insulin receptor in the presence of supernatant removed from previously autophosphorylated IR stimulated with insulin in vitro . (A) Equal amounts of partially purified IR were incubated with 10-7M insulin for 60 minutes at 4 C and the incubation continued for 60 minutes at 22 C in the presence of buffer (lane 1,2) or 25μM unlabelled ATP (lane 4,5). The reaction was terminated by rapid cooling and the IR removed by immunoprecipitation with anti-human insulin receptor antibody (HIR) and insulin removed by anti-insulin antibody . Fresh, unstimulated IR's were (lane 2,5) or were not (lane 1,4) added to the supernatants and incubated in the presence of γ - 32P-ATP (25μM) to undergo autophosphorylation for 60 minutes at 22 C. Fresh, unstimulated IR in the absence of supernatant (lane3) and in vitro insulin (10-7 M) activated IR (lane6) were allowed to undergo autophosphorylation in the presence of γ-32P-ATP (25 μM). The reactions were terminated and the IR's were immunoprecipitated, resolved by SDS-PAGE and subjected to autoradiography as described under "Materials and Methods". There was no significant phosphorylation observed of the fresh receptors in the presence of any of the supernantants while insulin could normally stimulate these receptors. (B) Autophosphorylation band intensities from autoradiographs (Fig.13.A) were quantified by densitometry.

Figure 13 C

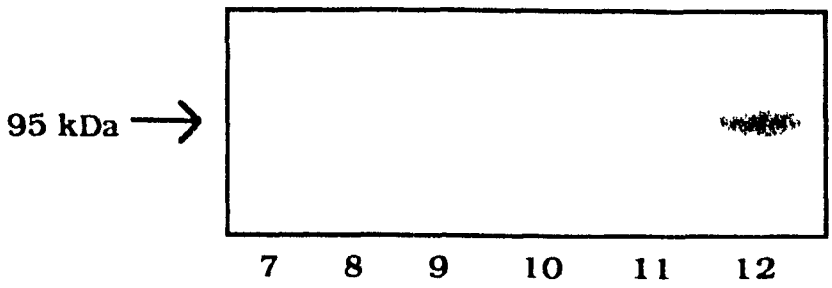


Figure 13 D

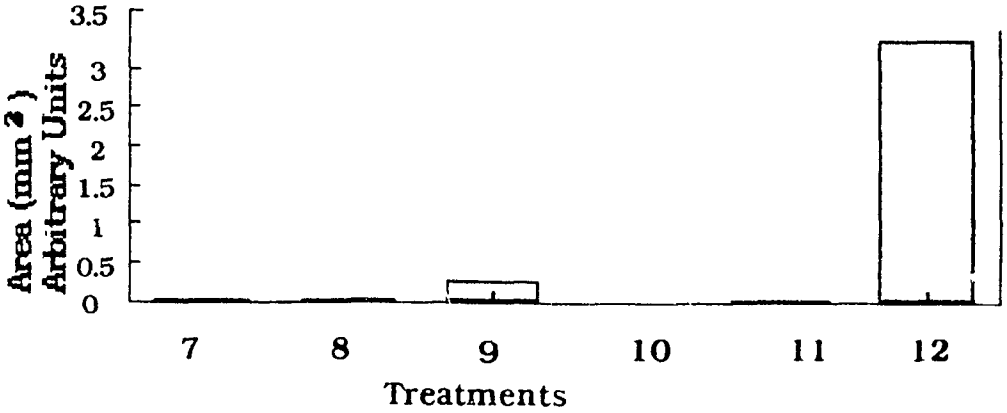


Fig 13.C&D. Autophosphorylation studies of unstimulated partially purified insulin receptor in the presence of supernatant removed from previously autophosphorylated IR stimulated with insulin In Vivo. (C) Lymphocytes were exposed to insulin for 1 minute at 37 C in vivo and IR subsequently purified as described in "Materials and Methods". These pre-activated partially purified IR were incubated with buffer (lane 7,8) or 25 μM unlabelled ATP (lane 10,11) for 60 minutes at 22 C . The reaction was terminated by rapid cooling and IR removed by immunoprecipitation with anti-human insulin receptor antibody (HIR). Fresh unstimulated IR's were (lane 8,11) or were not (lane 7,10) added to the supernatant and incubated in the presence of gamma-32P-ATP (25μM) to undergo autophosphorylation for 60 minutes at 22 C. Fresh unstimulated IR in the absence of supernatant (lane 9) and in vivo pre-activated IR (lane 12) were allowed to undergo autophosphorylation in the presence of γ-32P-ATP (25μM) in the absence of supernatant. The reactions were terminated and the IR's were immunoprecipitated, resolved by SDS-PAGE and subjected to autoradiography as described under "Materials and Methods". No significant phosphorylation of the unstimulated fresh receptor was observed in the presence of any of the supernatants. (D) Autophosphorylation band intensities from autoradiographs (Fig.13.C) were quantified by densitometry .

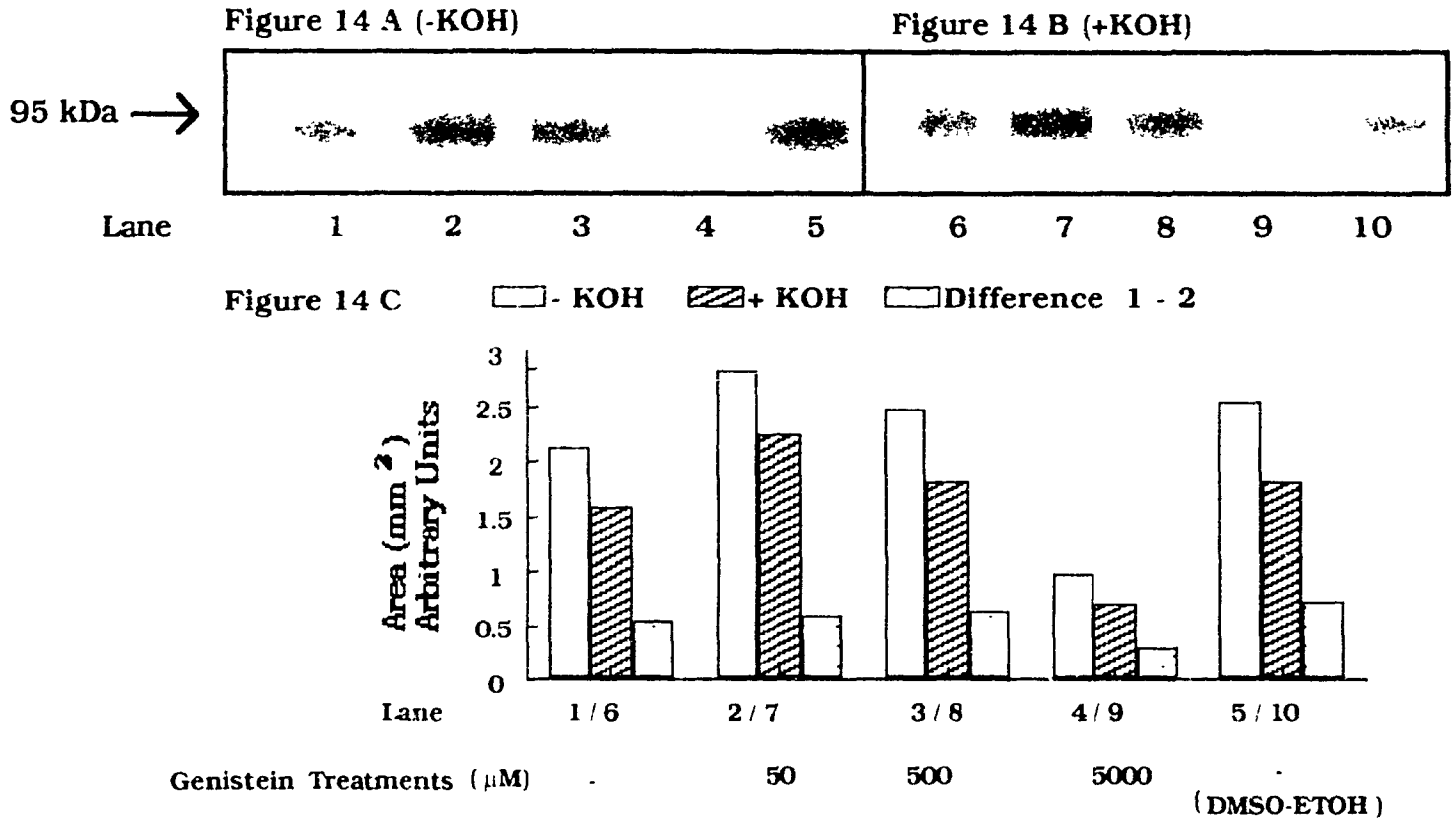


Fig.14.A&B&C. Effect of Genistein on insulin stimulated IR autophosphorylation. (A) Partially purified IR's were stimulated with 10-7M insulin in vitro in the presence of increasing concentrations of genistein (0-5000uM). Equal amounts of IR's were autophosphorylated, immunoprecipitated, resolved by SDS-PAGE and subjected to autoradiography as described in "Materials and Methods". (B) Gels from a parallel experiment were alkali washed and subjected to autoradiography as described under "Materials and Methods". (C) Autophosphorylation band intensities of autoradiographs (Fig.14.A&B) were quantified by densitometry. The difference between (-)KOH and (+)KOH intensities was taken to represent serine phosphorylation. DMSO-ETOH (lane 5&10) contained the maximum concentration of these dilutents used.

Figure 15 A

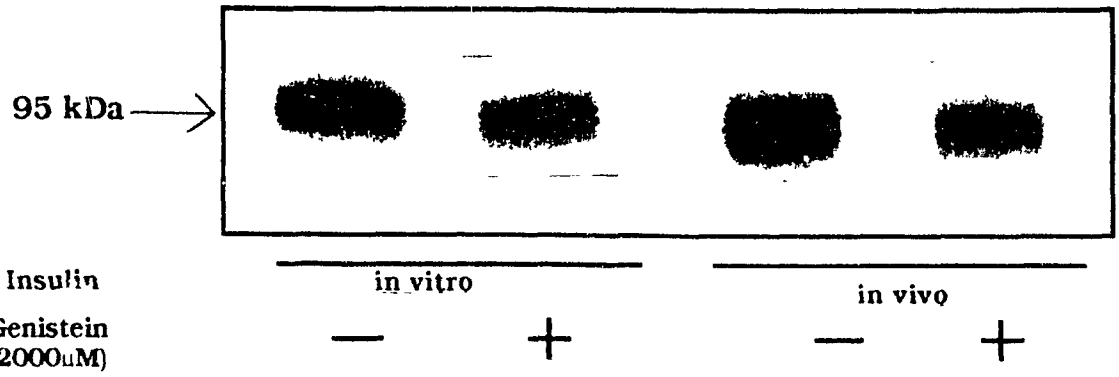


Figure 15 B

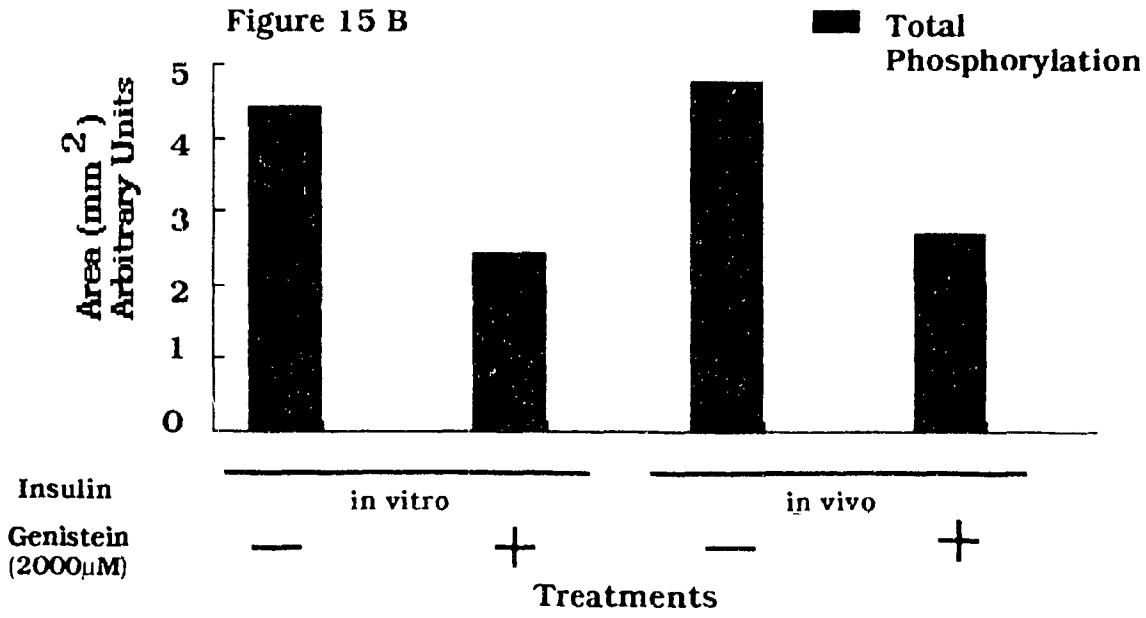


Fig 15.A&B. Effect of 2000uM Genistein on total phosphorylation of in vitro and in vivo insulin stimulated IR. (A) IM-9 lymphocytes were or were not stimulated with 10-7M insulin in vivo for 1 minute at 37 C. Partial purification of the IR followed and unstimulated IR were treated with 10-7M insulin in vitro for 60 minutes at 4 C. Equal amounts were autophosphorylated at 22 C in the presence or absence of 2000 uM genistein in vitro. The IR's were then immunoprecipitated, resolved on SDS-PAGE and subjected to autoradiography as described under "Materials and Methods". (B) Autophosphorylation band intensities from autoradiographs (Fig.15.A) were quantified by densitometry. Genistein significantly inhibited total IR phosphorylation.

(Fig.15.C&D). These data supported the close association of the required tyrosine phosphorylation for the serine phosphorylation and suggested the possibility that the serine phosphorylation may be catalyzed by the receptor tyrosine kinase itself.

One concern was the relatively high dose of genistein used which may have also inhibited a distinct serine kinase. The conditions of the autophosphorylation reaction were altered to allow an inhibitory effect to be observed at 50μg/ml (200 μM) genistein (Akiyama et al., 1987) by decreasing the gamma ^{32}P-ATP concentration from 25μM to 10μM and the time of autophosphorylation from 60 minutes to 15 minutes. Under these conditions, in which genistein has been documented to be specific for tyrosine kinases, there was an inhibitory effect on total ^{32}P incorporation of the in vivo activated insulin receptor (Fig.16A&B). However, similar to our previous results genistein exposure did not alter the ratio of ^{32}P-tyrosine to ^{32}P-serine consistent with the possibility that the phosphorylation of both amino acids may be catalyzed by the receptor enzyme (Fig.16C&D).

3.8 Effect of phosphatase inhibitors on insulin receptor phosphorylation .

Net insulin receptor ^{32}P incorporation depends on the balance of phosphorylation and dephosphorylation. Contamination of the IR preparation with either phosphotyrosine phosphatase or phosphoserine phosphatase activities may alter the relative incorporation (Le Marchand-Brustel et al., 1988). The autophosphorylation reaction was

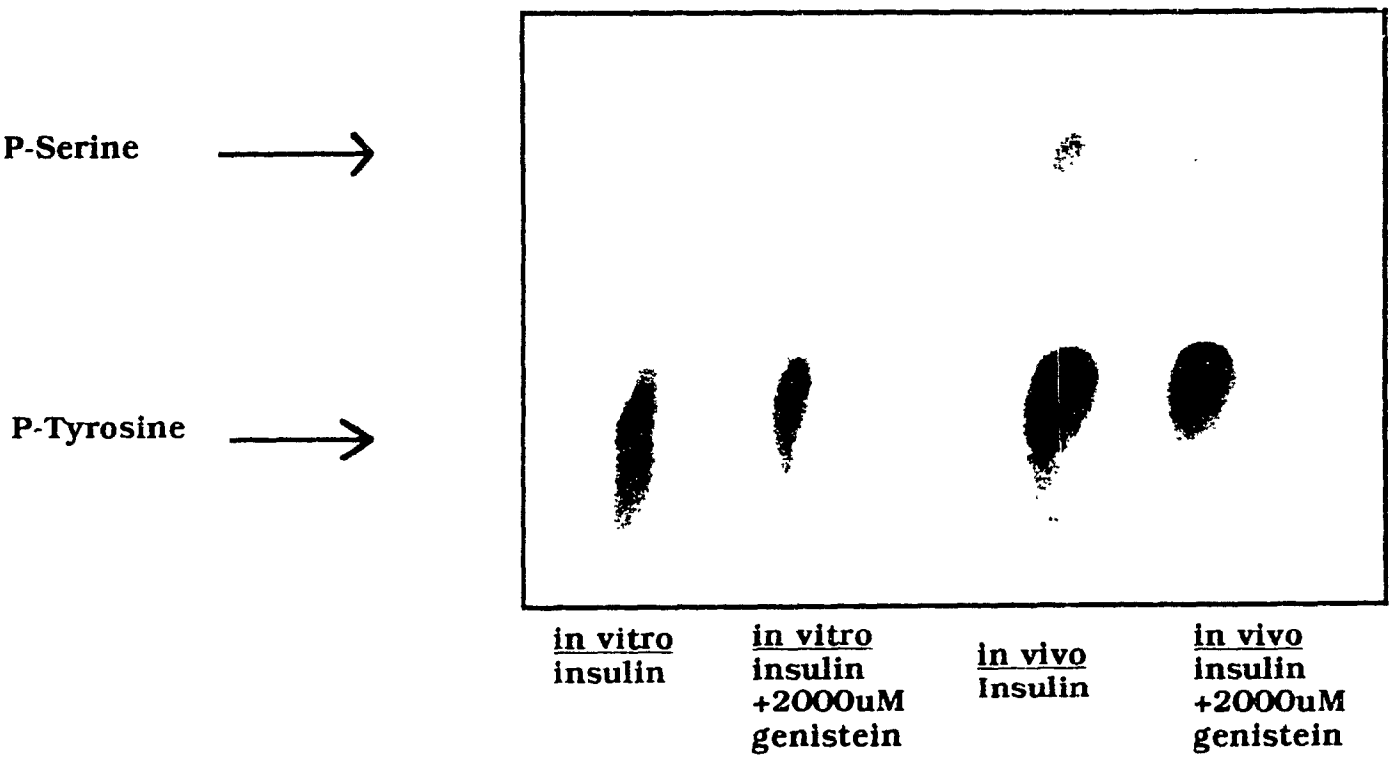


Fig.15.C. PAAA of 2000μM Genistein treated partially purified IR stimulated with insulin in vivo or in vitro. The 95-kDa band shown in Fig.15.A was excised from the gel and the radiolabelled protein was extracted and hydrolyzed as described in "Materials and Methods". Phosphoaminoacids were resolved by Thin Layer Electrophoresis (TLE) and subjected to autoradiography. The positions of P-Tyrosine and P-Serine are noted (arrows). The P-Tyrosine and P-Serine positions were quantified by LKB ultrascan XL enhanced laser Densitometer with two dimensional analysis.

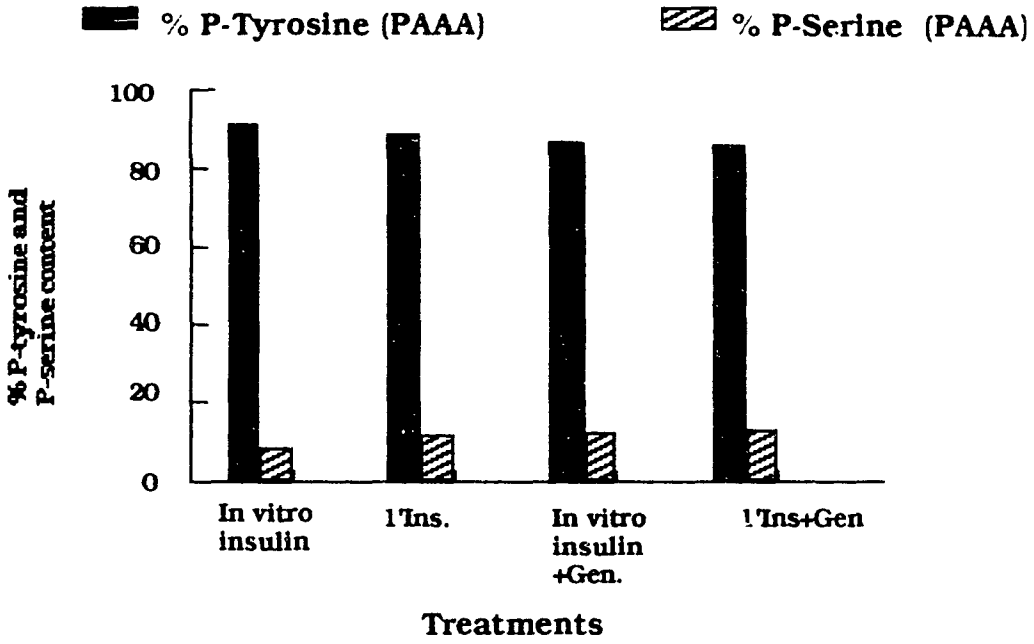


Fig.15.D. Quantitation of % P-Tyrosine and P-Serine content of partially purified IR stimulated with insulin in vivo or in vitro phosphorylated in the presence of 2000uM genistein in vitro. Genistein did not alter the ratio of phosphorylation of tyrosine to serine.

Figure 16A

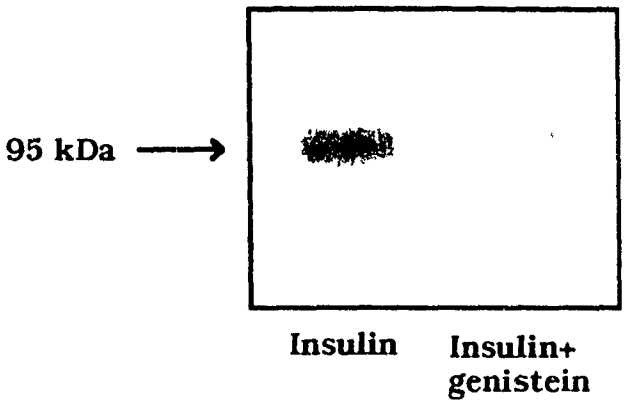


Figure 16B

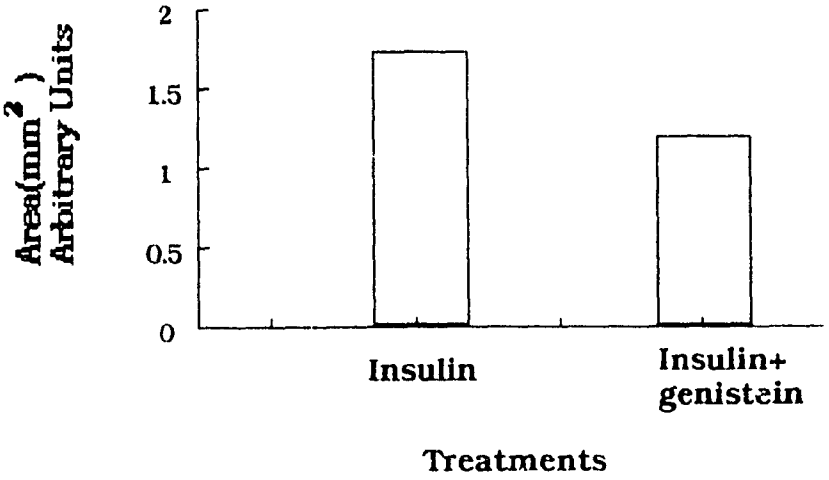


Fig.16.A&B. Effect of 50 μg/ml Genistein on total phosphorylation of partially purified IR stimulated with insulin in vivo. (A) IM-9 lymphocytes were stimulated by insulin in vivo (1' insulin). Partial purification of the IR followed and equal amounts of IR were autophosphorylated for 15 minutes in the presence of 10 uM γ - 32P - ATP without or with 50 μg/ml genistein, immunoprecipitated, resolved by SDS-PAGE and subjected to autoradiography as described under "Materials and Methods". (B) Autophosphorylation band intensities from autoradiographs (Fig.16.A) were quantified by densitometry.

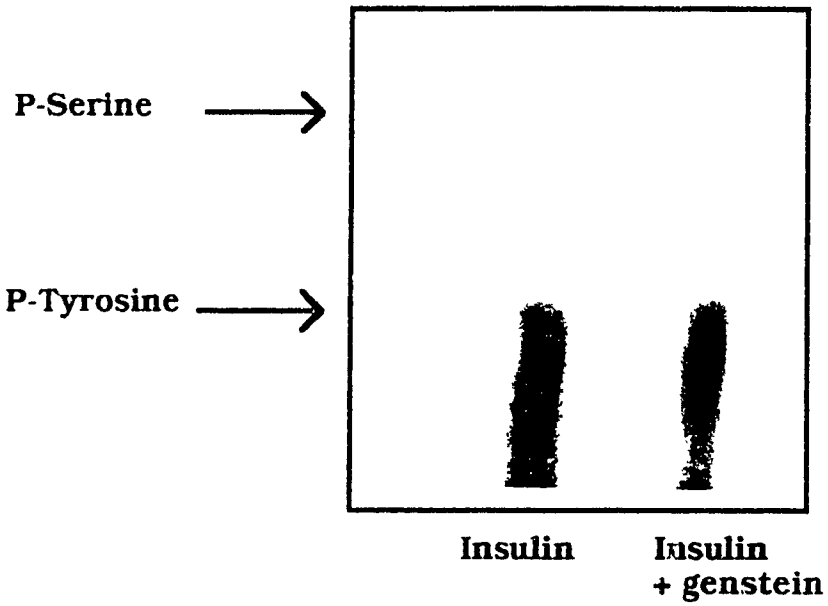


Fig.16.C. PAAA of partially purified IR stimulated with insulin in vivo and autophosphorylated in the presence and absence of 50ug/ml genistein. The 95-kDa band shown in Fig.16.A was excised from the gel and the radiolabelled protein was extracted and hydrolyzed as described in "Materials and Methods". Phosphoamino acids were resolved by TLE and subjected to autoradiography. The positions of P-Tyrosine and P-Serine are noted (arrows). The P-Tyrosine and P-Serine positions were quantified by LKB ultrascan XL enhanced laser densitometer with two dimensional analysis.

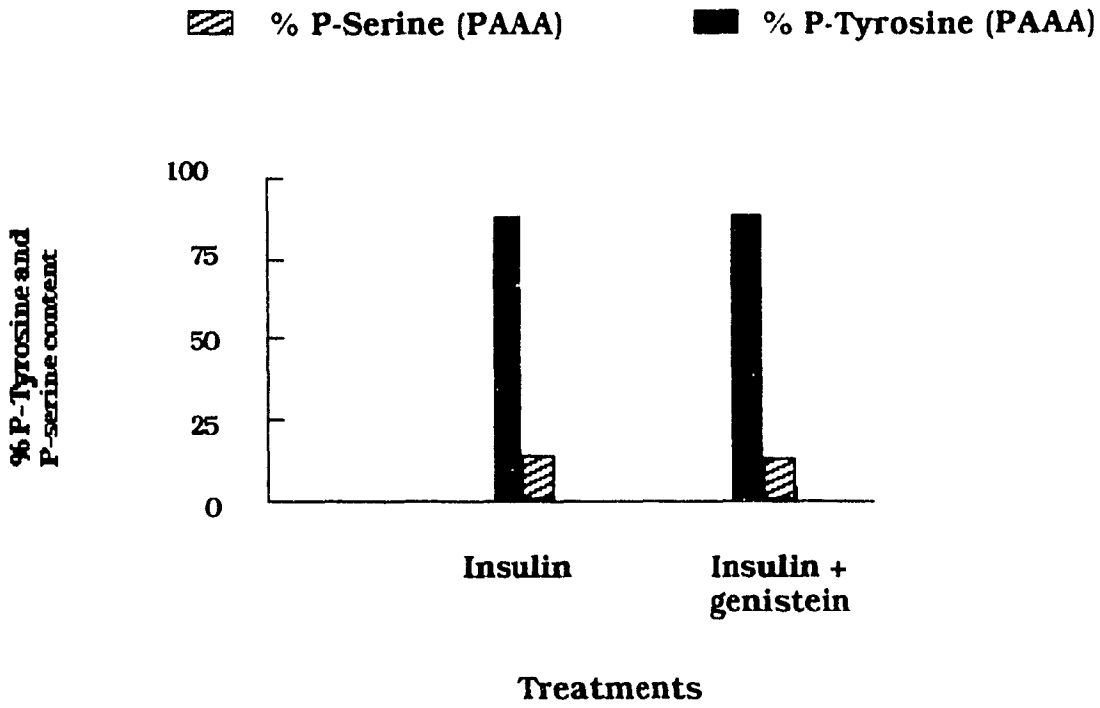


Fig.16.D. Quantitation of % P-Tyrosine and P-Serine content of phosphorylated partially purified IR stimulated with insulin in vivo and autophosphorylated in the presence and absence of 50μg/ml genistein in vitro.

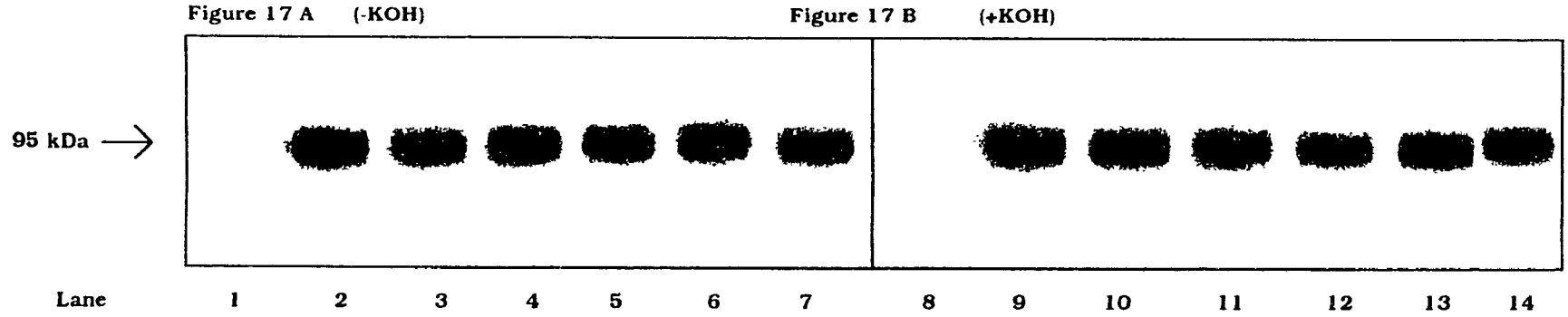
performed for 60 minutes at 22°C as described in the presence and absence of vanadate (up to 1666μM) or NaF (1.8mM) (Fig.17.A&B&C). These agents did not alter total phosphorylation nor the relative amounts of phosphotyrosine and phosphoserine.

3.9 Effect of divalent cation on tyrosine and serine phosphorylation .

It has been well documented that insulin receptor tyrosine autophosphorylation is $MnCl_2$ dependent (White et al., 1983; Nemenoff et al. 1984). Decreasing the concentration of $MnCl_2$ from 10mM to 1mM resulted in a dose-dependent decrease in total autophosphorylation (Fig.18A-C). This was associated with a decrease in both tyrosine and serine phosphorylation such that the relative incorporation of ^{32}P into these amino acids was not altered (Fig.18A-C). Thus, again these data support an intimate association between tyrosine and serine phosphorylation and support the possibility that the IR kinase itself may autophosphorylate on serine as well as tyrosine residues.

3.10 Effect of receptor concentration on tyrosine and serine phosphorylation .

The IR is known to exist predominantly as a heterotetramer, the two α-β subunit homodimers linked by disulfide bonds (Rosen, 1987). The investigation of tyrosine autophosphorylation revealed that since enzyme and substrate were the same molecule, the



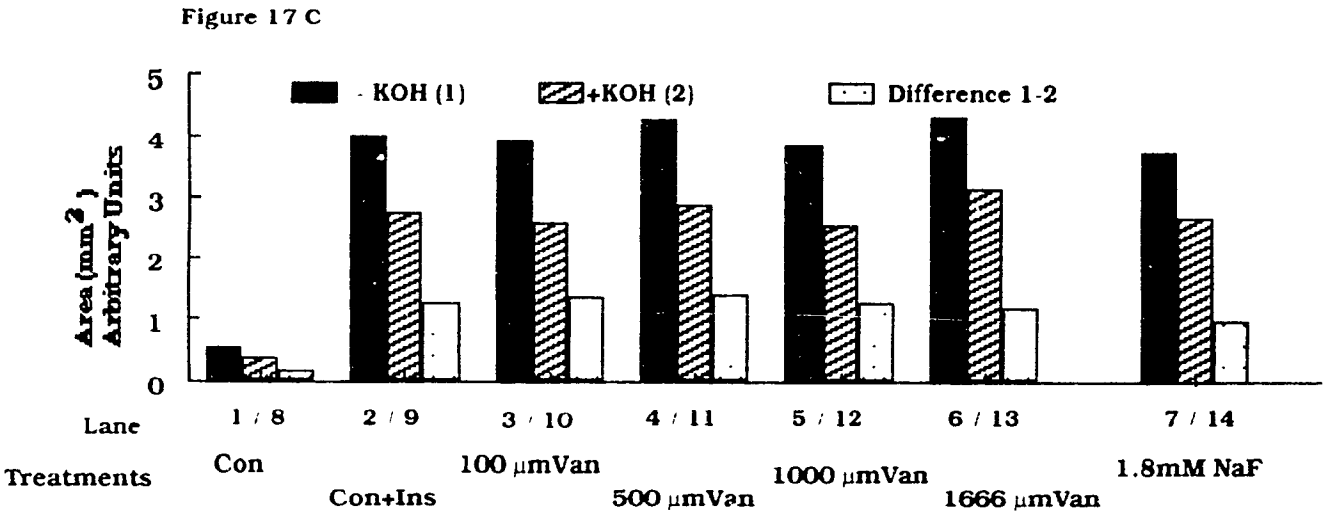


Fig.17.A&B&C. Effects of vanadate and sodium fluoride (NaF) on insulin receptor phosphorylation. (A) Equal amounts of specific binding of partially purified IR were stimulated with 10-7M insulin in vitro for 60 minutes at 4°C in the presence and absence of increasing concentrations of vanadate up to 1666 µM or 1.8 mM NaF. IR's were autophosphorylated, immunoprecipitated, resolved by SDS-PAGE and subjected to autoradiography as described under "Materials and Methods". (B) Gels of a parallel experiment were alkali washed and subjected to autoradiography as described under "Materials and Methods". (C) Autophosphorylation band intensities from autoradiograms (Fig.17.A&B) were quantitated by densitometry.

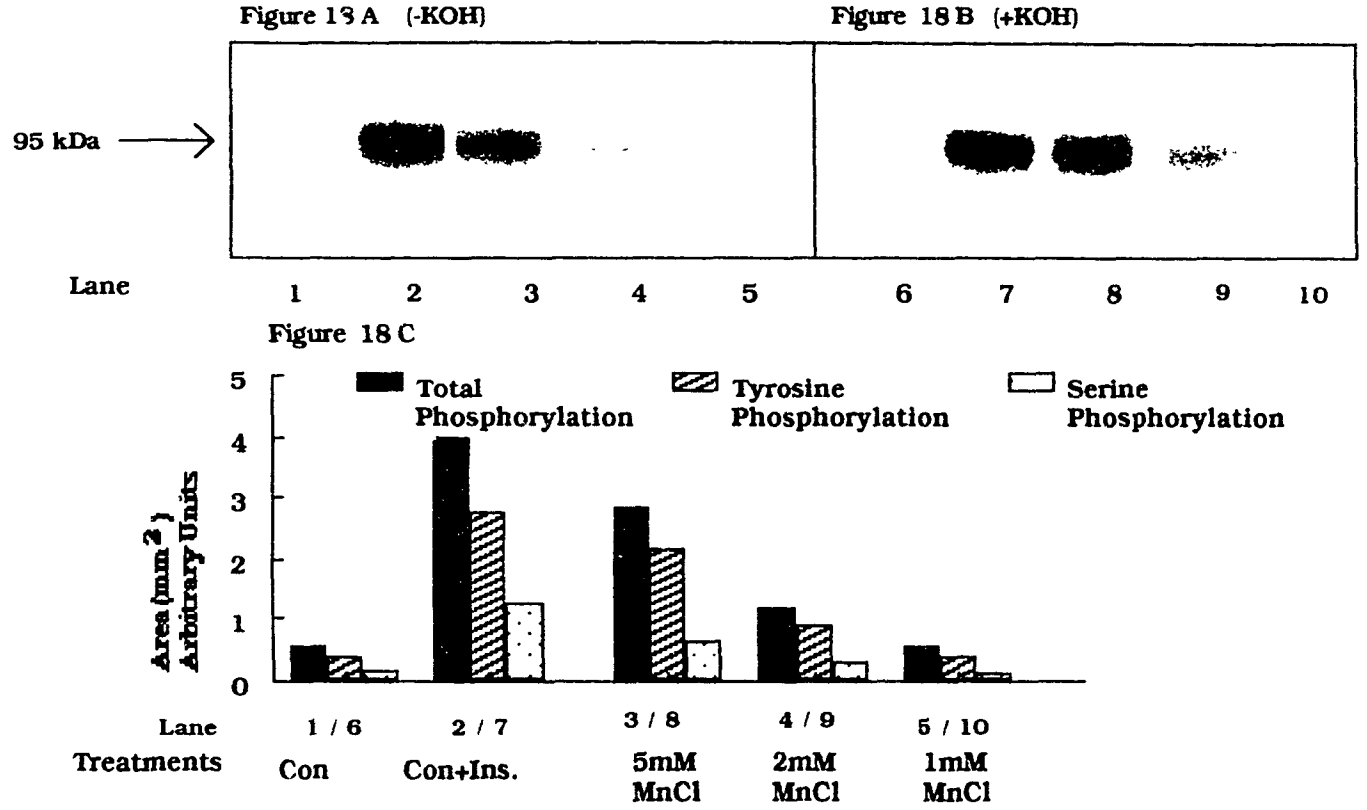


Fig.18.A&B&C. Effect of Manganese Chloride (MnCl2) concentration on insulin receptor phosphorylation. (A) Equal amounts of partially purified IR were stimulated with 10-7M insulin in vitro for 60 minutes at 4°C in the presence of decreasing concentrations of MnCl2 (5 , 2 , 1 mM). IR's were autophosphorylated, immunoprecipitated, resolved by SDS-PAGE and subjected to autoradiography as described under "Materials and Methods". (B) Gel of a parallel experiment were alkali washed and subjected to autoradiography as described under "Materials and Methods". Decreasing amounts of MnCl2 were associated with lower total phosphorylation but no alteration in the relative amount of KOH sensitive ^{32}P incorporation taken to represent serine phosphorylation. (C) Autophosphorylation band intensities from autoradiograms (Fig.18.A&B) were quantified by densitometry.

incorporation of ^{32}P-phosphate onto tyrosine (corrected for receptor concentration) did not change as the concentration of receptor was altered (Cobb et al., 1989). This indicated the intramolecular nature of the reaction. If phosphorylation occurs via an intermolecular reaction then dilution of the enzyme and the substrate containing preparation would lead to a decrease in the rate of phosphorylation. This observation would not be expected in an intramolecular or autophosphorylation reaction (Cobb et al. , 1989). To determine whether the extent of receptor serine phosphorylation varied in the same manner as tyrosine phosphorylation, the autophosphorylation reaction was carried out for 15 minutes, a time at which both amino acids were submaximally phosphorylated by in vitro insulin stimulation. The reaction was performed with the usual concentration of partially purified receptor (1X), half this concentration (0.5X) and twice the usual (2X). Total phosphorylation increased with receptor concentration but the ratio of ^{32}P-tyrosine and ^{32}P-serine remained constant over the entire range of receptor concentration (Fig.19.A-D). Correction for receptor concentration by ^{125}I-insulin binding curves revealed a constant relative amount of ^{32}P-tyrosine and ^{32}P-serine incorporation at all receptor concentrations (data not shown).

Another method to differentiate intramolecular from intermolecular reactions is to use an agent in the enzyme reaction which impairs the movement of molecules and their interactions thereby decreasing the rate of reaction (Cobb et al., 1989). Addition of 5% glycerol to the autophosphorylation reaction decreased total phosphorylation slightly (Fig.20A-D). Both tyrosine and serine

Figure 19 A

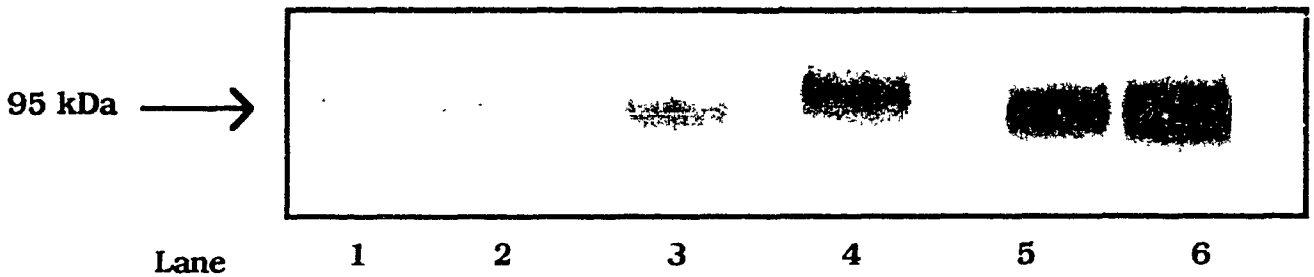


Figure 19 B

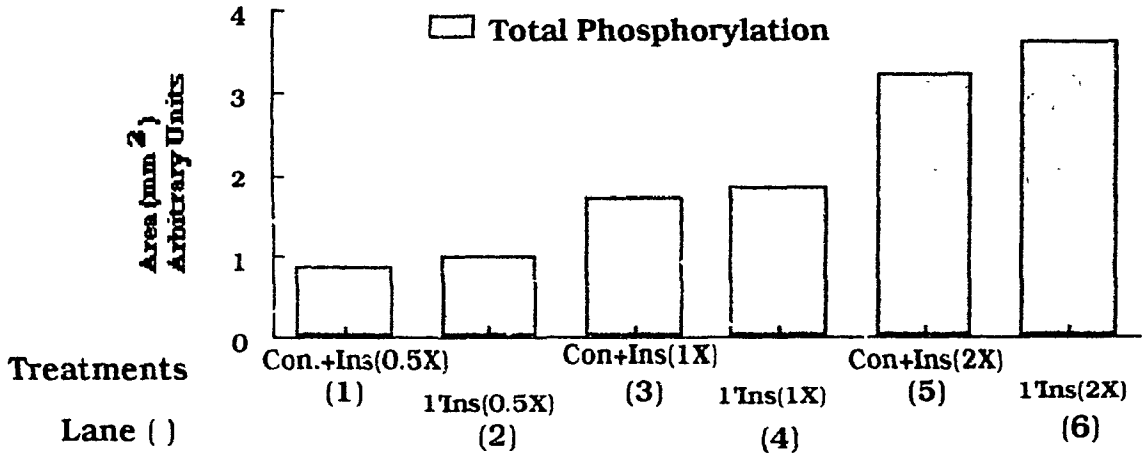


Fig.19.A&B. Effect of receptor concentration on total autophosphorylation. (A) IM-9 lymphocytes were stimulated with insulin in vivo for 1 minute at 37°C (lanes 2,4,6). Partial purification of the IR followed and unstimulated IR were stimulated with 10-7M insulin in vitro for 60 minutes at 4°C (lanes 1,3,5). Various amounts of IR at 0.5 , 1 and 2 times the concentration of receptor usually used were autophosphorylated, immunoprecipitated, resolved by SDS-PAGE and subjected to autoradiography as described under "Materials and Methods". Total phosphorylation was linearly correlated with receptor concentration. (B) Autophosphorylation band intensities from autoradiograms (Fig.19.A) were quantified by densitometry.

Fig.19.C. Effect of insulin receptor concentration on 32P incorporation into phosphoserine and phosphotyrosine of the IR (PAAA). The 95-kDa bands shown in Fig.19.A were excised from the gel and the radiolabelled proteins were extracted and hydrolyzed as described in "Materials and Methods". Phosphoamino acids were resolved by Thin Layer Electrophoresis (TLE) and subjected to radioautography. The positions of P-Tyrosine and P-Serine are noted (arrows). The P-Tyrosine and P-Serine positions were quantified LKB ultrascan XL enhanced laser densitometer with two dimensional analysis .Sample in lane 5 was lost due to technical factors.

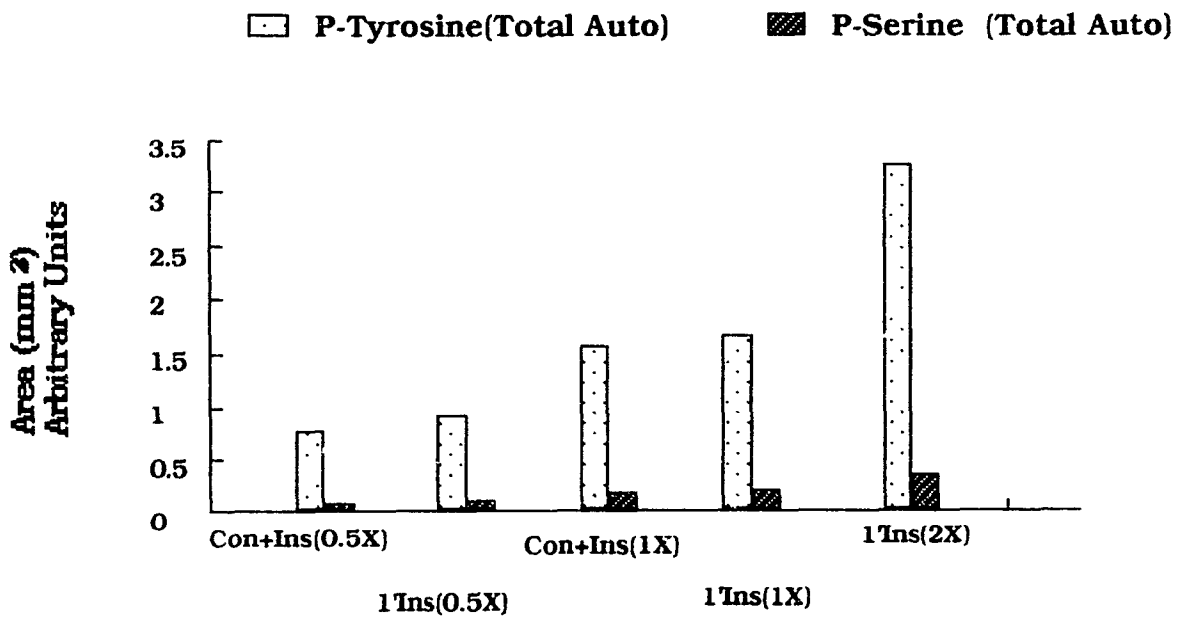


Fig.19.D. Total 32P-Tyrosine and 32P-Serine content of IR stimulated with insulin in vivo or in vitro phosphorylated at varying concentrations. Both tyrosine and serine phosphorylation were linearly correlated with receptor concentration. The relative amount of 32P-tyrosine and 32P-serine was not altered by the change in receptor concentration.

Figure 20 A

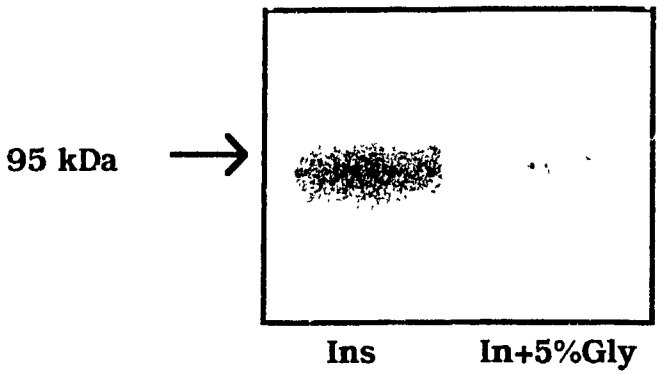


Figure 20 B

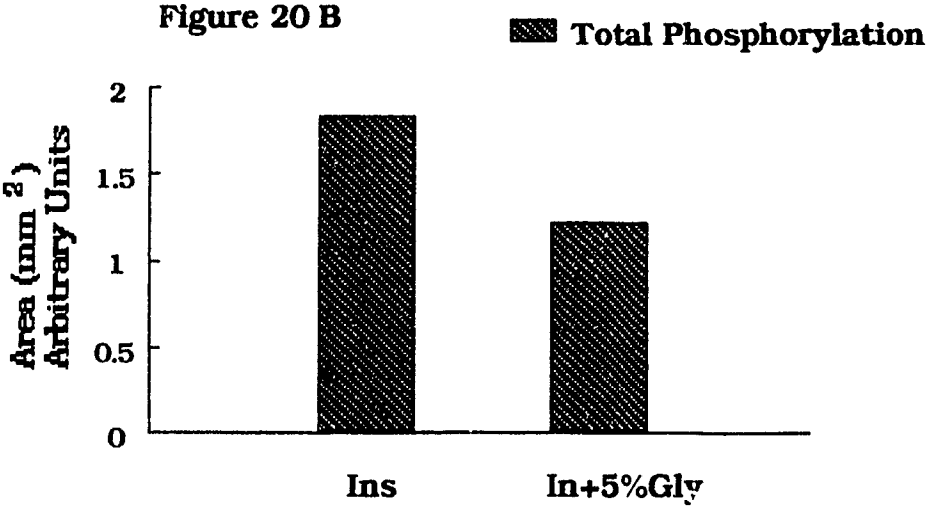


Fig.20.A&B. Effect of glycerol on insulin receptor phosphorylation. (A) IM-9 lymphocytes were stimulated with 10-7M insulin in vivo for 1 minute at 37°C. Partial purification of the IR followed and equal amounts of IR were incubated in the presence and absence of 5% (vol./vol.) glycerol autophosphorylated, immunoprecipitated, resolved by SDS-PAGE and subjected to autoradiography as described in "Materials and Methods". (B) Autophosphorylation band intensities from autoradiograms (Fig.20.A) were quantified by densitometry.

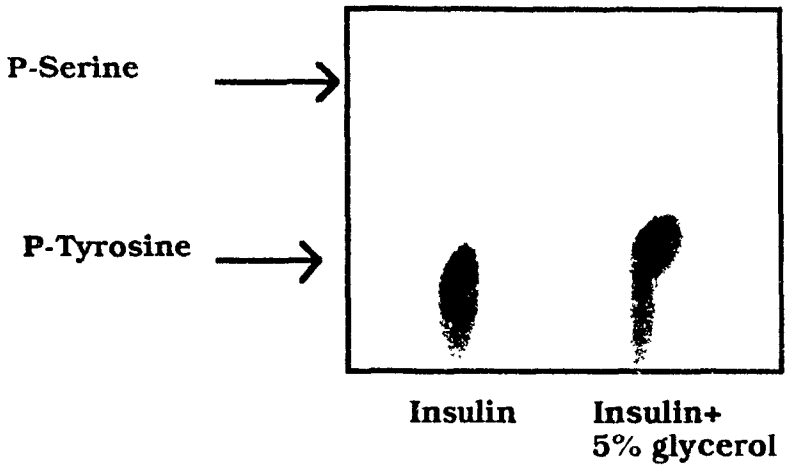


Fig.20.C. Effect of glycerol on 32P incorporation into phosphoserine and phosphotyrosine of the IR (PAAA). The 95-kDa band shown in Fig.20.A was excised from the gel and the radiolabelled protein was extracted and hydrolyzed as described in "Materials and Methods". Phosphoamino acids were separated by TLE and subjected to radioautography. The positions of P-Tyrosine and P-Serine are noted (arrows). The P-Tyrosine and P-Serine positions were quantified by a LKB ultrascan XL enhanced laser densitometer with two dimensional analysis.

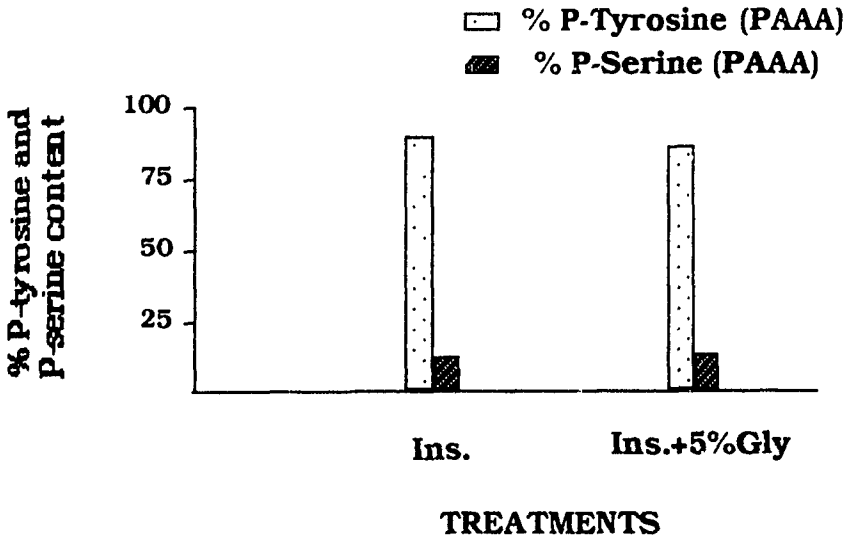


Fig.20.D. Quantitation of % 32P-Tyrosine and 32P-Serine of IR stimulated with insulin in vivo and phosphorylated in the presence of 5% glycerol.

phosphorylation were similarly affected. These data all support the hypothesis that receptor serine phosphorylation occurs by an intramolecular reaction. This would be consistent with either an autophosphorylation on serine or the presence of a distinct IRSK bound tightly and irreversibly to the receptor.

3.11 Consequence of serine phosphorylation on insulin receptor tyrosine kinase .

Previous studies demonstrated the association of IR serine phosphorylation with diminished tyrosine kinase activity (Takayama et al., 1984 & 1988; Haring et al., 1986; Karasik et al., 1990; Bollag et al., 1986). This was best shown with phorbol ester stimulated protein kinase C mediated IR phosphorylation. We confirmed in these studies (Fig.8. 9. 10) that PMA did cause a decrease in receptor kinase activity and autophosphorylation. To investigate whether the serine phosphorylation stimulated by insulin that we have demonstrated here can have any effect on the receptor kinase, insulin receptors were incubated with insulin under the conditions described for the autophosphorylation reaction at 22°C for various times. At the times indicated, aliquots of receptor were removed and a tyrosine kinase assay performed in the presence of labelled ATP (7μM) and the exogenous substrate poly Glu:Tyr (4:1). A a control, parallel incubations were performed at 4°C, a temperature at which serine phosphorylation does not occur (verified by PAAA). There was a gradual steady decrease of tyrosine kinase activity over time of the in vivo activated insulin receptor (Fig.21). The control receptor with

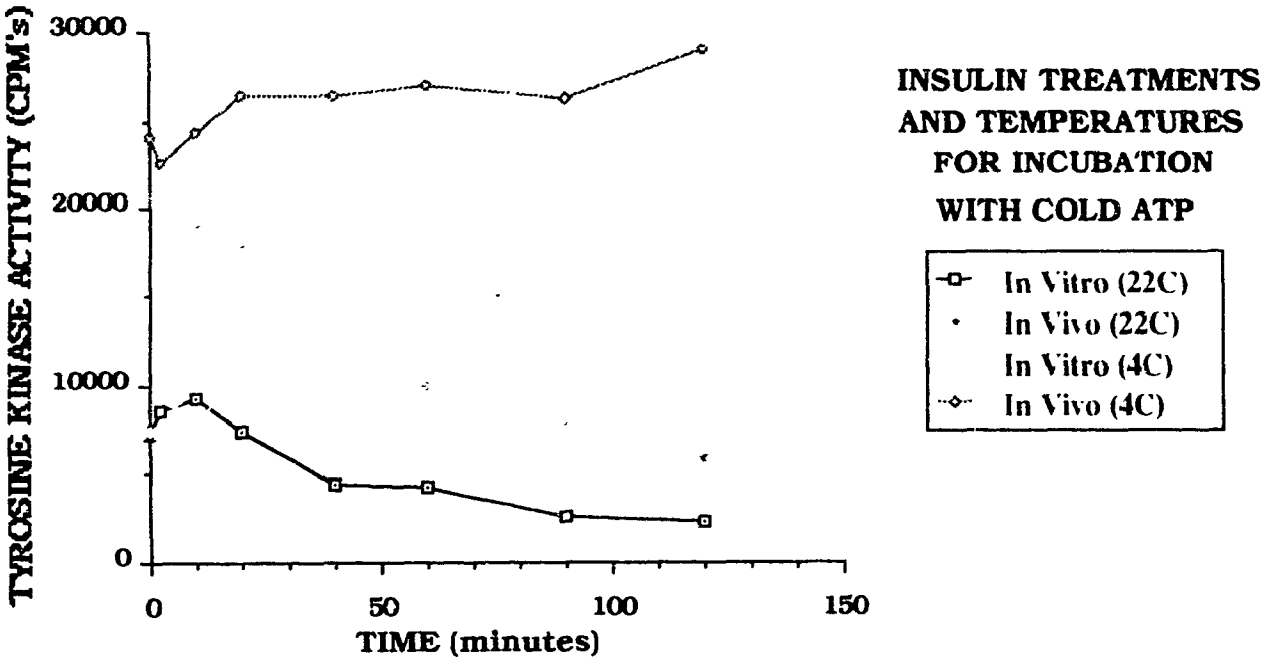


Fig.21. Effect of serine phosphorylation on the insulin receptor tyrosine kinase (IRTK) activity. IM-9 lymphocytes were or were not stimulated with 10-7M insulin in vivo for 1 minute at 37°C. Partial purification of IR followed and unstimulated IR were s .mulated with 10-7M insulin in vitro for 60 minutes at 4°C. Equal amounts of both receptors were pre-incubated with 25 μM unlabeled ATP for various times (0-120 minutes) at 4 or 22°C. At times shown above, aliquots were removed from the above incubation and placed into an IRTK assay containing poly Glu:Tyr (4:1) in the presence of 7μM γ-32P-ATP. The assay was performed for 30 minutes at 22°C and samples were counted as described in "Materials and Methods".

insulin added in vitro showed a biphasic curve with some increase in activity observed at 5 to 10 minutes followed by a decrease (Fig21). This suggests that activation of the IRSK at 22°C in this cell type inhibits the IRTK activity. The assay of IRTK activity was performed at 22°C but similar results were obtained with this assay at 4°C (data not shown).

To exclude the possibility that the loss of activity was not related to ATP depletion, the experiment was repeated in the presence of 500μM CTP and similar results were obtained (data not shown). With the in vivo activated insulin receptor incubated at 22°C, the IRTK activity at 0 time with addition of CTP was 15000 cpm's and decreased to 10000 cpm's after incubation for 60 minutes. As well, direct measurement of the concentration of ATP in the same preparation before and after 60 minutes of incubation revealed a decrease in ATP concentration from 25 μM to 18 μM ATP in the absence of CTP and no significant fall in the presence of CTP. These data indicate that ATP depletion was not responsible for the significant decrease in tyrosine kinase activity observed and are consistent with a possible regulatory role for insulin stimulated serine phosphorylation on the receptor tyrosine kinase.

DISCUSSION AND CONCLUSIONS

4.1 Existence of an insulin receptor serine kinase (IRSK)

In this study our goal was to identify and characterize the serine kinase activity catalyzing the phosphorylation of the insulin receptor (IR). We have clearly demonstrated that insulin stimulates insulin receptor serine kinase (IRSK) activity both in vitro, i.e. added directly to a partially purified insulin receptor preparation, and in vivo, i.e. in cultured human lymphocytes exposed to insulin (Fig.1. A, B, C). In both cases insulin stimulates tyrosine autophosphorylation which has been previousiy well documented (Kasuga et al., 1982 & 1983; Petruzzeli et al., 1984). The extent of activation of both activities tended to be somewhat greater in vivo, particularly after 1 minute of exposure of intact cells to insulin corrected for receptor concentration but this was not consistently observed. The extent of ^{32}P Phosphate incorporated into tyrosine was 80-90 % of total as compared to serine which comprised 10-20 % (Fig.2). Of interest throughout the study, we never observed threonine phosphorylation. This has been detected by others in placenta (Smith et al., 1988a) and hepatoma cell (Smith et al., 1988b) insulin receptors stimulated by insulin. We have no explanation for this difference except perhaps the different tissues involved.

These results were determined by quantitation of total phosphorylation of a 60 minute incubation of IR with labeled ^{32} P-γ-

ATP (25μM) and subsequent phosphoamino acid analysis (PAAA) of the hydrolyzed 95 kDa insulin receptor beta subunit excised from the SDS-gel, which yielded the relative ^{32}P-Tyrosine and ^{32}P-Serine content. Another simpler method which correlated well and indicated the approximate extent of tyrosine and serine phosphorylation was to quantify the intensities of the 95 kDa IR beta subunit bands in radioautographs of gels with and without treatment with KOH. This alkali wash has been documented to remove the more labile phosphate from serine and threonine residues while phosphotyrosine tends to be resistant (Weller, 1979).

4.2 Insulin stimulated IRSK time course

As a first attempt to characterize the serine kinase activity, we determined the in vivo activation time course. This revealed that both tyrosine and serine phosphorylation activities peaked at 1 minute of exposure and decreased by 30 to 60 minutes (Fig.3. A, B, C). Phosphoamino acid analysis of the identical in vivo activation time course confirmed that as total phosphorylation decreases with time (Fig.4. A, B), IRSK activity mirrors the effect of insulin receptor tyrosine kinase activity to phosphorylate their respective amino acids (Fig.4. C, D). This indicates that a close association exists between these two enzyme activities. Previous studies by other investigators suggested the existence of an insulin receptor serine kinase activity which was activated by the insulin receptor tyrosine kinase activity (Lewis et al., 1990b; Sale et al., 1988).

The decrease in tyrosine and serine phosphorylation after 30-60 minutes of exposure to insulin in vivo may be due to sites on these amino acids already phosphorylated in the intact cells and occupied by unlabeled phosphate or may reflect a true decrease in enzyme activity. Since insulin stimulated IR tyrosine autophosphorylation is known to be the mechanism for activation of the receptor tyrosine kinase, we performed western blots with anti-phosphotyrosine antibodies on the receptor that had been exposed to insulin in vivo for various times. Peak in vivo phosphotyrosine content was evident at 1 minute and fell significantly by 30 minutes returning to basal levels at 60 minutes (Fig.5. A, B). These data suggest that the decreased tyrosine autophosphorylation over time is not due to increased phosphotyrosine in vivo receptor content but correlates more with decreased phosphotyrosine content. The lack of a similar decrease to basal levels of tyrosine phosphorylation activity at 60 minutes when in vivo phosphotyrosine content returned to basal levels is not clear but may indicate that the activation of the receptor enzyme is influenced by other changes, e.g. receptor conformation, which may be more slowly reversed or that minimal tyrosine phosphorylation is required for activation. The close association of the serine kinase activity suggests that the decrease over time is related to the tyrosine kinase activity but this remains to be proved by studies of in vivo labeling of the IR in these cells.

Our in vivo studies and previous reports suggested that receptor tyrosine kinase activity was required to stimulate an insulin receptor serine kinase (IRSK) (Yu et al., 1984 ; Rosen et al., 1983).

To investigate this further, we studied the time course of incorporation of ^{32}Phosphate onto tyrosine and serine residues in vitro in the presence of insulin and after insulin activation in vivo. With in vitro insulin, tyrosine phosphorylation was detectable by 1 minute and increased, reaching maximum by 20 minutes. In contrast serine phosphorylation was not detectable at 1 or 3 minutes and appeared at 5 minutes, increasing to maximum at 20 minutes. With the in vivo pre-activated receptor, tyrosine phosphorylation was increased 5 fold at 1 minute of the assay and reached maximum by 5 minutes. As well, serine phosphorylation was detected at 1 minute and also reached maximum at 5 minutes (Fig.6. A, B). These data strongly suggest the requirement of receptor tyrosine phosphorylation for IRSK activation. This may indicate that the IRSK is activated by the insulin receptor tyrosine kinase (IRTK) or that the receptors can only serve as substrates once they are tyrosine phosphorylated or both. Lewis suggested that since IRSK activation can be assayed with a peptide substrate representing the presumed serine phosphorylation sites of the insulin receptor, that true activation of the IRSK occurs (Lewis et al., 1990).

4.3 Characterization of the IRSK and its close association with the insulin receptor and the insulin receptor tyrosine kinase (IRTK)

Previous investigations suggested that the IRSK is a distinct enzyme separate from the IR (Kasuga et al., 1982 ; Yu et al., 1987a; Gazzano et al. , 1983; Zick et al., 1983a ; Ballotti et al., 1986). In fact,

Smith showed that a wash of the WGA column with 1 M NaCl prior to elution of the IR decreased the serine phosphorylation (Smith et al., 1988). In this study we found that this procedure decreased slightly total receptor phosphorylation (Fig.7. A, B) but did not alter the relative phosphorylation on tyrosine and serine residues (Fig.7. C, D). This was also found by Czech (personal communication). NaCl may not interfere with the association of the IRSK to bind to the column or the receptor. Either the different purification methods of others or the presence of more than one serine kinase activity that can phosphorylate the insulin receptor may explain these discrepant results.

A distinct serine kinase which has been demonstrated to phosphorylate the IR on serine is protein kinase C (PKC) (Takayama et al., 1984; Haring et al., 1986a). Several reports have suggested that in many cell types insulin activates PKC (Cooper et al., 1987; Farese et al., 1986 & 1988). We found that PKC activation by a phorbol ester, phorbol-12-myristate-13-acetate (PMA), did not result in subsequently measured IRSK activity indicating that activation by insulin is unlikely to be mediated by PKC (Fig.8. A - D). However, PMA decreases the ability of insulin to stimulate the insulin receptor (Fig.9. A, B ; 10. A - D) as has been reported previously in adipocytes and rat hepatoma cells indicating that PKC was activated and phosphorylated the IR in vivo. Of interest, while total phosphorylation of the insulin stimulated receptor was decreased by PMA, the relative tyrosine and serine phosphorylation was not altered (Fig.3. A - D).

The requirement of IR tyrosine phosphorylation and lack of an easily separable IRSK indicated that the IRSK was tightly associated with the receptor. Immunoprecipitation of the phosphorylated receptor with anti-phosphotyrosine antibody demonstrated that all of the phosphorylation on serine occured on receptors phosphorylated on tyrosine (Fig.11. A, B, C, D). This is contrary to the findings of Magre et al. (Magre et al., 1989). They found in cultured human fibroblasts, two separate receptor populations, one which was serine phosphorylated and the other tyrosine phosphorylated. We also found that the remaining population of receptors, 85% of the initial concentration, was inactive and not stimulable by insulin.

In an attempt to isolate an IRSK activity, the IR's were allowed to undergo tyrosine and serine phosphorylation and then removed by immunoprecipitation with anti-human insulin receptor antibody. When fresh control (unstimulated) receptor was re-added to the supernatant as substrate, no IRSK activity was detected (Fig.13. A - D). Although it might be argued that these receptors required tyrosine phosphorylation to serve as effective substrates, the data of Lewis with the insulin receptor carboxyl-terminus peptide suggested that this was not the case (Lewis et al., 1990). An alternative explanation that we have considered is that the IRSK is the insulin receptor itself and that both tyrosine kinase activity and serine kinase activity are activated by receptor tyrosine autophosphorylation. This would explain the requirement of some receptor tyrosine phosphorylation prior to the onset of serine phosphorylation, the rapid activation of both activities in vivo and the coordinate inhibition of both activities by PMA.

To test this hypothesis several approaches were used. Firstly, the effect of the relatively specific tyrosine kinase inhibitor Genistein (Akiyama et al., 1987) was tested and was found to inhibit both activities to a similar extent (Fig.14. A - C ; 15. A - D ; 16. A - D).

4.4 Various effects of phosphatase inhibitors and divalent cations

Attempts to alter either tyrosine or serine phosphorylation by adding vanadate, a phosphotyrosyl phosphatase inhibitor, or NaF, a phosphoseryl phosphatase inhibitor, were not successful, perhaps since these phosphatases were not present in significant amounts in our insulin receptor preparation (Fig.17. A - C). In contrast, progressive lowering of the concentration of $MnCl_2$ from 10mM to 5mM to 1mM decreased total phosphorylation (Fig.18. A - C). Of significance, both tyrosine and serine phosphorylation were decreased to a similar extent. The key role of Mn in IR tyrosine autophosphorylation has previously been well documented (Yu et al., 1987c) and these results could suggest that this IRSK activity is also manganese dependent .

4.5 Evidence for serine phosphorylation via an intramolecular reaction

A method to determine whether an enzyme serves as it's own substrate in an intramolecular reaction is to assess the effect of enzyme concentration on the rate of the reaction (Cobb et al., 1989).

The autophosphorylation of the IR on tyrosine residues has been demonstrated to be an intramolecular reaction (Petruzzelli et al., 1984). We demonstrated in this study that the rate of IR serine phosphorylation is not altered by increasing or decreasing receptor concentration, strongly suggesting that this reaction is also catalyzed in an intramolecular fashion (Fig.19. A - D). This result may be due to either a distinct IRSK bound to the IR in a relatively irreversible fashion or the IRSK may be the IR itself. Our further studies using 5% glycerol (Cobb et al., 1989), which diminished total phosphorylation slightly but did not alter the relative incorporation of ^{32}P into tyrosine and serine, support this conclusion (Fig.17. A - D).

4.6 Evidence for IRSK regulation of the IRTK

Finally, we tested the possibility that the serine phosphorylation stimulated by insulin resulted in inhibition of tyrosine kinase activity. Previous studies with PMA (Takayama et al., 1984; Haring et al., 1986a) and isoproterenol (Haring et al., 1986b) or cAMP analogs suggested this type of regulation. Incubation of IR which had been activated in vivo or in vitro under conditions known to result in serine phosphorylation resulted in a decrease in exogenous tyrosine kinase activity indicating that serine phosphorylation may have an important regulatory role in insulin action (Fig. 21). This may play a part in the known effect of insulin to induce desensitization of target tissues to itself.

In conclusion, we have demonstrated that insulin stimulates IR serine phosphorylation as well as tyrosine phosphorylation. These two activities are intimately related and at least some tyrosine phosphorylation precedes serine phosphorylation. The two enzyme activities : 1) increase and decrease in parallel under a variety of conditions, 2) be inseparable by elution from each other with 1 M NaCl or by immunoprecipitation and 3) involve serine phosphorylation only on tyrosine phosphorylated receptors. In addition, changing receptor concentration or incubation with 5% glycerol do not alter the ratio of phosphotyrosine to phosphoserine. All these data support the concept that the IR is the IRSK. An exogenous IRSK would have to be irreversibly bound to the receptor to explain these findings. This alternative seems much less likely.

Of interest, there are recent reports that there exist proteins that exhibit cross specificity for tyrosine and serine/threonine phosphorylation. The protein in question is the fission yeast $p107^{wee1+}$ mitotic inhibitor. This yeast enzyme is capable of autophosphorylation on both tyrosine and serine residues (Featherstone et al., 1991). There exists as well a mammalian enzyme, Clk from erythroleukemia cells, which is related to the cell cycle regulating kinases (cdc 2-like) which has catalytic activity for phosphorylation of both tyrosine and serine residues (Ben-David et al., 1991). However, the IR is the first tyrosine kinase to have the possible function of phosphorylating serine. Since other serine/threonine kinases are known to phosphorylate the IR and result in decreased tyrosine kinase activity, our data suggesting that

the insulin stimulated serine phosphorylation may also be associated with a diminished tyrosine kinase activity suggest that this phosphorylation may not be merely spurious or promiscuous. Czech has also recently presented data suggesting that IR serine phosphorylation is catalyzed by the IR itself (Czech, 1991). At the recent International Diabetes Federation Meeting, Czech presented his hypothesis that the IR is it's own serine kinase. This was based on evidence that there was high stoichiometry of phosphorylation of serine which paralleled that of tyrosine phosphorylation. Similar to our results, he has not been able to separate an IRSK from the receptor by various physical and biochemical techniques (personal communication). It would not be inconceivable for the IR to have several mechanisms for its regulation by different serine kinases. This would allow for dampening of the insulin signal in various physiological states and redundancy of an important homeostatic mechanism to maintain normal concentrations of blood glucose.

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