Activation of Protein Kinase Cα Inhibits Signaling by Members of the Insulin Receptor Family*

(Received for publication, May 4, 1995, and in revised form, July 13, 1995)

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Stimulation of the activity of protein kinase C by pretreatment of cells with phorbol esters was tested for its ability to inhibit signaling by four members of the insulin receptor family, including the human insulin and insulin-like growth factor-I receptors, the human insulin receptor-related receptor, and the Drosophila insulin receptor. Activation of overexpressed protein kinase Cα resulted in a subsequent inhibition of the ligand-stimulated increase in antiphosphotyrosine-precipitable phosphatidylinositol 3-kinase mediated by the kinase domains of all four receptors. This inhibition varied from 97% for the insulin receptor-related receptor to 65% for the Drosophila insulin receptor. In addition, the activation of protein kinase Cα inhibited the in situ ligand-stimulated increase in tyrosine phosphorylation of the GTPase-activating protein-associated p60 protein as well as Shc mediated by these receptors. The mechanism for this inhibition was further studied in the case of the insulin-like growth factor-I receptor substrate. PI, phosphatidylinositol; PKC, protein kinase C; IGF, insulin-like growth factor; IGFR, IGF receptor; IRR, insulin receptor-related receptor; hIR, hIGFR, and hIRR, human IR, IGFR, and IRR.

Most studies have documented a critical role for the intrinsic tyrosine kinase activity of the insulin receptor (IR) in mediating subsequent biological responses (1, 2). After binding insulin, the receptor autophosphorylates on several specific tyrosines and then tyrosine-phosphorylates several endogenous substrates including insulin-receptor substrate (IRS)-1, an SH2-containing protein called Shc, and various 60-kDa proteins including one that is tightly bound by the GTPase-activating protein of Ras (3–10). The tyrosine phosphorylation of IRS-1 results in its being bound by several SH2-containing proteins including the phosphatidylinositol (PI) 3-kinase, a tyrosine phosphatase, and two SH2 linker proteins called Grb-2 and Ndk (3, 11). A number of studies utilizing a variety of approaches have implicated the tyrosine phosphorylation of IRS-1 as being important in initiating several biological responses including stimulation of growth responses as well as stimulation of glucose uptake (12–17).

Because of the critical role of the receptor tyrosine kinase in initiating subsequent biological responses, a major question is how this receptor kinase activity may be regulated. Interest in this question is also stimulated by the finding that the IR kinase may be negatively regulated in patients with non-insulin-dependent mellitus, possibly contributing to the insulin resistance observed in this condition (18–25). Negative regulation of the IR kinase in several cell systems by a variety of different types of prior treatments has also been reported; for example, treatment of cells with high concentrations of insulin for long periods of time, with tumor necrosis factor, with activators of protein kinase C or cyclic AMP, or with inhibitors of Ser/Thr phosphatases and even incubation of the cells with high concentrations of glucose have all been reported to decrease the ability of insulin to stimulate the IR kinase activity (26–33). In most of these cases, the detailed biochemical mechanism whereby these agents elicit the inhibition is still not known. In one of the best understood systems, pretreatment of adipocytes with the Ser/Thr phosphatase inhibitor, okadaic acid, an increase in the Ser/Thr phosphorylation of IRS-1 was observed, which inhibited its subsequent ability to be tyrosine-phosphorylated in vitro by the IR and to be bound by the PI 3-kinase (32). However, it is not clear which kinase is responsible for this hyperphosphorylation of IRS-1 and which specific Ser/Thr residues in IRS-1 are responsible for its decreased ability to be tyrosine-phosphorylated.

Another system that has been extensively studied is the ability of activated protein kinase C (PKC) to antagonize IR signaling. This system is particularly attractive, since several reports have indicated that hyperglycemia can result in activation of PKC (34, 35). Although some studies have indicated that activation of PKC increases the Ser/Thr phosphorylation of the IR and this phosphorylation inhibits its ability to subsequently tyrosine-phosphorylate in vitro various exogenous substrates; diR, Drosophila IR; PMA, phorbol 12-myristate 13-acetate; GAP, GTPase-activating protein; CHO, Chinese hamster ovary; HBS, Hepes-buffered saline.
strates (29, 36), other studies do not confirm this finding (30, 37–40). Activation of overexpressed PKCα was observed to stimulate the Ser/Thr phosphorylation of the IR and to inhibit the in situ insulin-stimulated increase in tyrosine phosphorylation of the endogenous substrate IRS-1 and its subsequent binding by PI 3-kinase even under conditions in which no decrease in receptor kinase activity could be detected in in vitro assays utilizing exogenous substrates (30, 38). In addition, a number of specific Ser/Thr residues in the IR have been identified as phosphorylation sites both in vitro and in vivo. These include two serines (Ser 1303/1306 and Ser 1327) and one threonine (Thr 1348) in the carboxy tail of the IR, serines in the juxtamembrane region of the IR (Ser 967/968), and serines in the kinase domain of the IR (Ser 1035/1037) (41–49). The role of these Ser/Thr phosphorylation sites in the IR in mediating the PKC-induced inhibition is not clear since mutant IR lacking these Ser/Thr phosphorylation sites still exhibited decreased signaling abilities after activation of PKC (46, 47, 50).

In order to further elucidate the mechanism whereby activation of PKC inhibits subsequent signaling by the IR, we set out in the present study to examine the ability of overexpressed PKCα to antagonize signaling by the other members of the IR family, including the human receptor for insulin-like growth factor I (hIGFR), the human insulin receptor-related receptor (hIRR), and the Drosophila IR (dIR). These three receptors differ considerably in their amino acid sequences from the human IR (51–53). For example, the carboxy tail of dIR lacks the two serine and one threonine phosphorylation sites present in human IR (52), whereas the dIR juxtamembrane and kinase domains are only 29 and 64% identical to the respective regions of the human IR (53). Despite these considerable differences in amino acid sequence, in the present study we show that the ligand-stimulated increase in PI 3-kinase mediated by all four receptors is inhibited by the activation of PKCα, although the extent of inhibition did differ to some extent. Moreover, in the present report we show that the ligand-stimulated increase in tyrosine phosphorylation of the GAP-associated p60 as well as the adaptor protein Shc mediated by these receptors is also inhibited by activation of PKC. Finally, the inhibition of IRS-1 and p60 tyrosine phosphorylation observed in situ could not be replicated in vitro by the use of IRS-1 or p60 from PKC-activated cells. These results support the hypothesis that the inhibition observed with PKC activation is important in modulating signaling by all members of the IR family and indicates that this inhibition is not caused by an increased Ser/Thr phosphorylation of these substrates.

**EXPERIMENTAL PROCEDURES**

Materials—The following were purchased: protein G-Sepharose beads from Pharmacia Biotech Inc., protein A-agarose beads from Repligen, wheat germ agglutinin coupled to agarose from EY Laboratories Inc., horseradish peroxidase-conjugated anti-phosphotyrosine antibody BC2A from Transduction Laboratories, peroxidase chemiluminescence detection kit (ECL) from Amersham Corp., purumycin and phenyl phosphate from Sigma, cell proliferation assay kit (MTS/PMS) and alkaline phosphatase-conjugated anti-mouse or anti-rabbit antibody from Promega, polyclonal anti-IRS-1 antibody and polyclonal anti-Shc antibody from UBI, phorbol 12-myristate 13-acetate (PMA) from Calbiochem, and l-phenylphthaldinisol from Avanti Polar Lipids, Inc. The monoclonal antibody to the GAP-associated 60-kDa protein (2C4) was as described previously (9). The following were gifts: monoclonal anti-IRS-1 antibody (1D3) from Dr. Kazu Yonezawa (Massachusetts General Hospital), anti-phosphotyrosine antibody PY20 from Dr. J. Glenney (University of Kentucky), polyclonal antibody to GAP from Dr. Jay Gibbs (Merck), cDNA encoding PKCα from Dr. Peter Parker (Ludwig Institute for Cancer Research), anti-PKCα antibody from Dr. W. Heath (Lilly and Co.), and the cDNA-encoding Drosophila insulin receptor from Dr. R. Fernandez (New York University).

Cell Lines—CHO cells stably overexpressing bovine PKCα (38) were transfected with either the cDNA encoding the human IR (hIR) (54), hIGFR (55), a chimera consisting of the extracellular portion of the human IR and the intracellular domain of the human IRR (hiR/hIRR) (56), or a chimera consisting of the extracellular portion of the human IR and the intracellular domain of the Drosophila IR (hiR/dIR) (57). Transfections were performed by a modification of the method of Chen and Okajama (58) as described previously (38). After drug selection, individual clones were picked and screened by Western blotting for PKCα expression. Those with high receptor and PKC levels were further subcloned by limiting dilution.

Immunoprecipitations and Western Blots—CHO-PKC cells overexpressing hIGFR, hIR, hIR/hIRR, or hIR/dIR were incubated in serum-free Ham's F-12 with 20 mM Hepes, pH 7.6, for 30 min at 37°C. Cells were washed with or without final concentration 1 μM insulin, followed by a 10-min ligand stimulation (final concentration 1 μM insulin). Cells were washed once with ice-cold 20 mM Hepes, pH 7.5, buffered saline (HBS) and lysed with lysis buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 20 μg/mL g-mercaptoethanol, 1 mM sodium orthovanadate). The lysates were cleared by centrifugation and incubated with 20 μl of protein G-Sepharose beads previously coated with 5 μg of either monoclonal antibody 2C4 to GAP-associate p60 (9), monoclonal anti-IRS-1 antibody 1D6, or control mouse IgG or protein A-agarose coated with anti-Shc antibodies. After 4 h at 4°C, the beads were washed three times with HBS containing 0.1% Triton X-100 (HBST), heated to 100°C for 3 min in SDS sample buffer, and the eluted samples analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting with horseradish peroxidase-conjugated anti-phosphotyrosine antibody BC2A. Bound antibodies were visualized with the ECL kit. In some experiments, after exposure to film, RC20 was stripped off the membrane by washing with 10 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Tween 20, 100 mM phenyl phosphate, and the membrane was reblotted with either the polyclonal antibodies to IRS-1 (dilution 1:1 μg/ml) or Shc (1 μg/ml). Bound antibodies were detected with an alkaline phosphatase-conjugated secondary antibody and a colorimetric substrate for alkaline phosphatase.

In Vivo Phosphorylation of p60 and IRS-1—100-mm plates of confluent CHO-PKC cells overexpressing the human IGF receptor were washed with ice-cold HBS and lysed in 750 μl of lysis buffer for 30 min at 4°C. Aliquots of 20 μl were used as described above. The lysates were centrifuged, and the supernatants were incubated with 20 μl of protein G-Sepharose beads that had been coated with monoclonal anti-p60 antibody BC2A, monoclonal anti-IRS-1 antibody 1D3 or control mouse IgG. After 4 h at 4°C, the immunoprecipitates were washed three times with HBST and then incubated for 20 min at room temperature with partially purified IGF receptor in the presence of 1 μM insulin, 2 mM MgCl2, 2 mM MnCl2, 2 mM ATP in 20 mM Hepes, pH 7.5, 0.1% Triton X-100. The beads were washed three times with HBST, and the samples were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting with horseradish peroxidase-conjugated anti-phosphotyrosine antibody BC20 and detection by the ECL kit. To prepare the partially purified IGF receptor, lysates from a 100-mm plate of confluent CHO-hIGFR cells were used with 40 μl of wheat germ agglutinin-agarose beads for 3 h at 4°C. After washing three times with HBST, the IGF receptor was eluted from the beads with 40 μl of HBST containing 0.3 mM N-acyetylglucosamine. Ten microliters of this preparation were used for each reaction.

Cell Proliferation Assays—5 × 104 cells were plated in a 96-well plate in serum-free Ham's F-12 supplemented with 0.1% bovine serum albumin, 20 mM Hepes, 100 units/ml penicillin, and 100 μg/ml streptomycin and treated with or without 0.5% PMA (final concentration 1 μM) and concurrently with the indicated concentrations of IGF-I. Cells were grown for 48 h at 37°C, 20 μl of phenazine methosulfate were added to the cells, and they were incubated at 37°C for 2 h. The absorbance at 490 nm was measured in a microtiter plate reader.

PI 3-Kinase Assay—PI 3-kinase activity was assayed as described (59). In brief, cells in 100-mm plates were incubated in serum-free Ham's F-12 with 20 mM Hepes (pH 7.6) for 2 h at 37°C, 1 μM PMA was added, and after 20 min at 37°C, ligand (1 μM insulin) was added. After 10 min at 37°C, the cells were washed once with ice-cold HBS and lysed in 1% SDS, pH 8.0, lysis buffer, and after 10 min, 100 mM Tris, pH 8.0, 137 mM NaCl, 2 mM MgCl2, 1 mM CaCl2, 10% glycerol, 1% Nonidet P-40, 0.4 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride. The lysates were centrifuged, and the supernatants were incubated with 20 μl of protein G-Sepharose beads previously coated with the monoclonal anti-phosphotyrosine antibody PY20 (2 μg/sample). After 4 h at 4°C, the beads were washed twice with each of the following buffers: 1) phosphate-buffered saline, pH 7.6, 0.5 mM LiCl, 1 mM dithiothreitol; 2) 100 mM Tris, pH 7.6, 0.5 mM NaCl, 1 mM dithiothreitol; and 3) 10 mM Tris, pH 7.6, 0.1 mM NaCl, 1 mM dithiothreitol. The beads were incubated for 5 min on ice.
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in 20 μl of buffer 3, and then 20 μl of 0.5 mg/ml phosphatidylinositol (previously sonicated in 50 mM Heps, pH 7.6, 1 mM EGTA, 1 mM NaH2PO4) was added. After 5 min at room temperature, 10 μl of the reaction buffer were added (50 mM MgCl2, 100 mM Hepes, pH 7.6, 250 μM ATP containing 5 μCi of [γ-32P]ATP), and the beads were incubated for an additional 5 min. The reactions were stopped by the addition of 15 μl of 4 n HCl and 130 μl of chloroform:methanol (1:1). After vortexing for 30 s, 30 μl from the phospholipid-containing chloroform phase was spotted onto thin layer chromatography plates previously coated with 1.3% potassium oxalate, 2 mM EDTA in H2O:methanol (3:2) and baked at 110°C for at least 3 h before spotting. The plates were placed in tanks containing chloroform:methanol:NH4OH:H2O (600:470:20:113) for 40-50 min until the solvent reached the top of the plates. The plates were dried at room temperature and autoradiographed, and the PI 3-phosphate spots were cut out and counted.

RESULTS

To study the interactions of PKCa with the different members of the IR family, we isolated stably transfected CHO cell lines overexpressing PKCa and these four different receptors. For hIRR and dIR, chimeric receptors were utilized that contained the extracellular domain of the human IR and the cytoplasmic domains of these two receptors (56, 57). These different cell lines (called CHO-PKC-hIR, CHO-PKC-hIGFR, CHO-PKC-hIR/hIR, and CHO-PKC-hIR/dIR) for the cells overexpressing the hIR, the hIGFR, the chimeric hIR/hIRR, and the chimeric hIR/dIR, respectively) each expressed comparably elevated levels of PKCa in comparison with CHO-hIR cells (data not shown) and elevated levels of the transfected receptors (the relative amounts of these four receptors could not be directly compared since different antibodies were required to visualize the different receptors). Activation of the overexpressed PKCa by a 20-min pretreatment with the phorbol ester PMA was found to inhibit the subsequent ligand-stimulated increase in anti-phosphotyrosine-precipitable PI 3-kinase mediated by all four receptors, whereas this treatment had no significant effect on CHO-hIR cells, which do not overexpress PKCa (Fig. 1). The inhibitions observed were 70, 80, 97, and 65% (average of four different experiments) for the cells overexpressing PKCa and the human IR, IGFR, IRR, and Drosophila IR, respectively. For each receptor, the results were confirmed with a second independent clone of cells. Again, the cells overexpressing the hIRRR exhibited a greater inhibition than the hIR, whereas the cells overexpressing the Drosophila IR exhibited slightly less inhibition than the hIR.

The above described differences in inhibition between the various members of the IR family suggested that the PKC effects may be mediated via an effect of this Ser/Thr kinase on the receptors themselves. If this were true, one might expect that activation of the PKCa would also inhibit the receptor-mediated tyrosine phosphorylation of other substrates. To test this, the various cell lines were again pretreated with PMA and then stimulated with ligand, and the extent of tyrosine phosphorylation of another endogenous substrate was measured, the p60 GAP-associated protein (9). PMA pretreatment was found to inhibit the ligand-stimulated increase in tyrosine phosphorylation of this substrate in the CHO cells overexpressing all four members of the IR receptor family and PKCa but not the control cells, which overexpressed the hIR but not PKCa (Fig. 2A). The tyrosine phosphorylation of p60 was likely to be mediated by the overexpressed IR family member in each of these cell lines since the extent of tyrosine phosphorylation of this protein was in all cases increased over that observed in cells overexpressing only PKCa (Fig. 2A). In these cells, unlike another previously described cell line (60), PMA treatment alone did not stimulate the tyrosine phosphorylation of p60 (data not shown).

The ligand-stimulated increase in tyrosine phosphorylation of a third endogenous substrate, Shc, (5, 8) was also examined in these cells. The tyrosine phosphorylation of Shc was increased in the cells overexpressing the hIR, hIGFR, and hIR/hIRR (but not in the cells expressing hIR/dIR) over that observed in the parental cells and in the cells overexpressing only PKCa (Fig. 2C). In each of these cells, activation of the overexpressed PKCa was found to inhibit the ligand-stimulated increase in tyrosine phosphorylation of Shc (Fig. 2C). As with the other two substrates, the insulin-stimulated increase in tyrosine phosphorylation of Shc was not inhibited in the cells containing only the endogenous levels of PKC (Fig. 2C).

To further study the mechanism whereby PKC activation inhibits signaling, the ability of the isolated IGF1 was examined for its capacity to phosphorylate in vitro IRS-1 that had been isolated from either control cells or cells in which PKCa had been previously activated (Fig. 3). No significant difference was observed in the extent of IRS-1 phosphorylation in vitro by isolated IGF1 (in three experiments, the ratio of tyrosine phosphorylation of IRS-1 isolated from PMA-treated cells to phosphorylation of IRS-1 isolated from control cells was 1.1 ± 0.25). In contrast, in parallel experiments, the IGF1-stimulated tyrosine phosphorylation of IRS-1 that occurred in situ was decreased by about 70% after PMA pretreatment (Fig. 3) (the ratio of IRS-1 tyrosine phosphorylation in PMA-treated cells/control cells was 0.28 ± 0.18), a value that is in good agreement with the 80% decrease in the IGF1-stimulated increase in anti-phosphotyrosine-precipitable PI 3-kinase (Fig. 1).

We also compared the ability of the IGF1 to phosphorylate in vitro p60 from either PMA-treated or control cells. The extent of p60 phosphorylation in vitro was not significantly decreased if the p60 was isolated from PMA-treated cells versus control cells (Fig. 2B). Again, in contrast, in parallel plates the in situ tyrosine phosphorylation of p60 was greatly inhibited by prior activation of the PKCa (Fig. 2A).

To determine whether activation of PKCa would affect the
ability of the IGF-I to mediate a biological response, CHO-PKC-IGFR cells were treated with or without PMA, and then the ability of IGF-I to stimulate proliferation was measured. PMA pretreatment was found to greatly inhibit the ability of IGF-I to stimulate the proliferation of these cells (Fig. 4).

**DISCUSSION**

In the present studies, activation of overexpressed PKCα was found to antagonize the signaling abilities of four distinct members of the IR family including the human IR and IGFIR, the orphan receptor called IRR and the Drosophila IR (Fig. 1). The sequences of these four receptors differ considerably (51–53), although their signaling abilities all appear to be quite similar (Refs. 55–57 and the present study). The inhibition occurred at one of the earliest steps mediated by these receptors, the ligand-stimulated increase in anti-phosphotyrosine-precipitable PI 3-kinase, a monitor of IRS-1 tyrosine phosphorylation (3). In addition, activation of the overexpressed PKCα was found to inhibit the ligand-stimulated increase in tyrosine phosphorylation of p60. The p60 was immuno precipitated from either control or PMA-treated CHO-PKC-IGFR cells and then incubated with either activated IGFR or buffer, as indicated. Control precipitations were performed with normal Ig (NM) and also incubated with IGFR. The samples were then analyzed by SDS gel electrophoresis and immunoblotting with anti-phosphotyrosine antibodies. C, in situ tyrosine phosphorylation of Shc. The different CHO cells were treated as in A and lysed, and the Shc was immunoprecipitated and analyzed by SDS gel electrophoresis and immunoblotting with an anti-phosphotyrosine antibody. The position of the major Shc band is indicated.

**FIG. 3.** In situ versus in vitro phosphorylation of IRS-1. Parallel plates of CHO-PKC-IGFR cells were pretreated with PMA or buffer and then either utilized for the in situ or in vitro phosphorylations. For the in situ phosphorylations, the cells were stimulated with ligand, and the IRS-1 was immunoprecipitated and analyzed by immunoblotting with anti-phosphotyrosine antibodies. For the in vitro phosphorylations, the IRS-1 was immunoprecipitated from cells and then incubated with either activated IGFR or buffer, as indicated. The samples were then analyzed by SDS gel electrophoresis and immunoblotting with anti-phosphotyrosine antibodies. The total amount of IRS-1 immunoprecipitated from the different cells was also analyzed and found to be the same by stripping the blot and reprobing it with a polyclonal anti-IRS-1 antibody. Ppt ab, precipitating antibody.

**FIG. 4.** Effect of PMA-treatment on IGF-I-stimulated proliferation of CHO-PKC-IGFR cells. Cells were treated with the indicated concentrations of IGF-I in the presence or absence of 1 μM PMA. Proliferation was assessed by the use of the phenazine methosulfate assay. Results shown are means ± S.E. for five experiments, and the results have been expressed as a percentage of the proliferative response observed in the presence of 1 nM IGF-I.

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phosphorylation of two other endogenous substrates, the GAP-associated p60 (9) (Fig. 2A) as well as the adaptor protein Shc (4, 5) (Fig. 2C). The tyrosine phosphorylation of IRS-1 and its subsequent association and activation of the PI 3-kinase has been found to be critical for subsequent signaling by the IR and presumably by the other members of this family (12–17). This effect of the activated PKCα could therefore explain its ability to inhibit the IGF-I-induced proliferative response in these cells (Fig. 4). Alternatively, PKCα could also be acting through an effect on a downstream molecule in the cascade, which leads to the proliferative response (61).

In the present studies, activation of the endogenous PKC was found to be insufficient to inhibit signaling by the overexpressed human IR in the CHO cells (Figs. 1 and 2). In contrast, in several prior reports activation of the endogenous levels of PKC have been reported to inhibit signaling via the IR, both in cells with and without overexpressed human IR (29, 33, 50). Some of these differences could be due to different levels of endogenous PKC in the various cell types studied as well as differences in the various isoforms of PKC present in these different cells. The ratio of PKC to IR may be critical in determining whether the inhibition of IR signaling is observed. In this regard, it would be important to determine whether activation of endogenous PKC in normal target tissues of insulin action is sufficient to inhibit insulin-stimulated increases in IRS-1-associated PI 3-kinase.

Activation of overexpressed PKCα has previously been found to stimulate a limited extent the Ser/Thr phosphorylation of IRS-1 protein (38). This increased phosphorylation of IRS-1 could have explained its decreased ability to be tyrosine-phosphorylated by the IR and the other members of this family. However, no decrease in the in vitro phosphorylation of IRS-1 by the IGFR was observed when the IRS-1 was isolated from cells in which the PKCα had been previously activated (Fig. 3). These findings differ from another cellular system of insulin resistance, okadaic acid-treated adipocytes (32). In this system, the Ser/Thr phosphatase inhibitor okadaic acid was observed to cause a hyperphosphorylation of IRS-1, which was evident from a shift in its mobility on SDS gel electrophoresis, and this hyperphosphorylation was reported to inhibit the subsequent in vitro phosphorylation of IRS-1 by the IR. Such a shift in IRS-1 has not been observed after the activation of PKCα (38), consistent with the lack of effect in the in vitro phosphorylation (Fig. 3). In addition, activation of the PKCα inhibited the in situ phosphorylation of the second endogenous substrate, the GAP-associated p60 protein, without affecting the in vitro phosphorylation of this substrate (Fig. 2). These results indicate that the PKCα is not causing its effect by stimulating the Ser/Thr phosphorylation of these two endogenous substrates.

This hypothesis is also supported by the finding that the extent of inhibition by PKCα varied to some extent for the four different receptors. Most notable was the greater inhibition of the signaling by hIR (Fig. 1). This receptor differs from the hIR in part by lacking 48 amino acids in the carboxyl tail of the β chain (52), including two previously identified serine phosphorylation sites as well as a threonine-phosphorylated residue in this region (41–43, 48). These results, therefore, indicate that phosphorylation of these residues in the carboxyl tail of the IR may not be important in mediating the negative regulation of the IR kinase; this conclusion is consistent with prior
studies that have indicated that Ser/Thr phosphorylations in the carboxyl tail region have little effect on the IR kinase (49, 50). Furthermore, sequencing the amino acid sequences of the cytoplasmic domains of these four receptors indicates that there are 11 conserved serines in all four molecules (Fig. 5). Only one of these serines (Ser-1190) is conserved in other receptor tyrosine kinases such as the epidermal growth factor receptor (62). The conserved serine in the juxtamembrane region of these four receptors (Ser-974) has been previously found by site-directed mutagenesis not to play a major role in mediating the PKC-induced inhibition of the IR kinase (46). It is therefore possible that the phosphorylation domain of one or a combination of these serine residues in the kinase domains of these receptors is responsible for the observed inhibitions.

The inhibition of the in situ tyrosine phosphorylation of IRS-1 and GAP-associated p60 caused by activation of PKCα is not reflected in a decrease in receptor kinase activity in in vitro assays (30, 38). Several hypotheses could explain these data. First, it is possible that the in vitro conditions do not replicate the conditions of the in situ situation. Second, it is possible that a third protein is involved in the intact cells. This protein may preferentially recognize the Ser/Thr phosphorylated IR and inhibit its kinase activity in situ; such a protein would function like arrestin, a protein that preferentially recognizes the serine-phosphorylated rhodopsin (63). Alternatively, it is possible that under nonstimulated conditions, a third protein tethers the IR together with its various substrates. After PMA-stimulated Ser/Thr phosphorylation, the IR may release this protein, thereby resulting in the decreased tyrosine phosphorylation of IRS-1 and other substrates. Such a protein would be similar to the yeast protein Ste5, which has been hypothesized to make a complex with various components of the yeast MAP kinase cascade, thereby facilitating their interactions (64).

Acknowledgments—We are grateful to Dr. Peter Parker for the cDNA encoding PKCα, Dr. John Glenny for Py20, Dr. Kazu Yonezawa for the monoclonal antibody to IRS-1, Dr. Rafael Fernandez for the Drosophila IR cDNA, Dr. W. Heath for the antibody to PKCα, and Dr. Lu-Hai Wang for the antibody to the IGFR.

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