In NIH-3T3 Fibroblasts, Insulin Receptor Interaction with Specific Protein Kinase C Isoforms Controls Receptor Intracellular Routing*

(Received for publication, December 29, 1997, and in revised form, February 13, 1998)

Pietro Formisano, Francesco Oriente, Claudia Miele, Matilde Caruso‡, Renata Auricchio, Giovanni Vigliotta, Gerolama Condorelli, and Francesco Beguinot§

From the Dipartimento di Biologia e Patologia Cellulare e Molecolare “L. Califano” and Centro di Endocrinologia ed Oncologia Sperimentale del Consiglio Nazionale delle Ricerche (CNR), “Federico II” University of Naples Medical School, Naples, Italy.

Insulin increased protein kinase C (PKC) activity by 2-fold in both membrane preparations and insulin receptor (IR) antibody precipitates from NIH-3T3 cells expressing human IRs (3T3HR). PKC-α, -δ, and -ζ were barely detectable in IR antibody precipitates of unstimulated cells, while increasing by 7-, 3.5-, and 3-fold, respectively, after insulin addition. Preexposure of 3T3HR cells to staurosporine reduced insulin-induced receptor coprecipitation with PKC-α, -δ, and -ζ by 3-, 4-, and 10-fold, respectively, accompanied by a 1.5-fold decrease in insulin degradation and a similar increase in insulin retroendocytosis. Selective depletion of cellular PKC-α and -δ, by 24 h of 12-O-tetradecanoylphorbol-13-acetate (TPA) exposure, reduced insulin degradation by 3-fold and similarly increased insulin retroendocytosis, with no change in PKC-ζ. In lysates of NIH-3T3 cells expressing the R1152Q/K1153A IRs (3T3Mut), insulin-induced coprecipitation of PKC-α, -δ, and -ζ with the IR was reduced by 10-, 7-, and 3-fold, respectively. Similar to the 3T3HR cells chronically exposed to TPA, untreated 3T3Mut featured a 3-fold decrease in insulin degradation, with a 3-fold increase in intact insulin retroendocytosis. Thus, in NIH-3T3 cells, insulin elicits receptor interaction with multiple PKC isoforms. Interaction of PKC-α and/or -δ with the IR appears to control its intracellular routing.

The turn on of the insulin signaling mechanism by the insulin receptor (IR)¹ involves a complex network of protein-protein interactions (1). Insulin-bound receptors phosphorylate a variety of docking proteins which include the IRS and Shc systems. Once phosphorylated, the docking proteins recruit and activate multiple insulin effectors (1). By employing the IRSs to engage Src homology 2 domain proteins, the IR avoids the stoichiometric constraints encountered by receptors which directly recruit these signaling molecules to their autophosphorylation sites (1, 2). The IRSs widen the connection and the tuning opportunities of the insulin signaling (3). There is evidence, however, that certain insulin bioeffects also follow the direct interaction of the IR with major insulin effectors. These effectors include phosphatidylinositol 3-kinase (4, 5) and, possibly, protein kinase C (PKC) (6, 7).

PKCs represent a family of structurally and functionally related serine/threonine kinases derived from multiple genes as well as from alternative splicing of single mRNA transcripts (8, 9). The individual isoforms differ in their regulatory domains and in their dependence on Ca²⁺, as well as in their tissue distribution and intracellular localization (10, 11). PKCs appear to play a dual role in the insulin signaling network. First, PKCs control insulin-dependent receptor kinase activation (12–15) and may regulate IRS-1 signaling as well (12, 13). Second, at least in certain cells and tissues, insulin activation of PKCs is required to evoke insulin effects on glucose transport and its intracellular metabolism (16, 17). Current evidence (6) indicates that chimeric receptors consisting of the EGF receptor extracellular domain fused to the cytoplasmic domain of the IR form stable complexes with PKC-α following EGF binding. It has also been reported that insulin increases PKC activity in Tyr(P) Ab precipitates from KB cells (22). However, the molecular mechanisms of PKC activation in response to insulin as well as the role of each individual PKC isoform in insulin signal transduction is still unsettled.

In previous reports (23) we demonstrated that Arg¹¹⁵² and Lys¹¹⁵³ in the regulatory domain of the IR kinase are crucial for enabling IR phosphorylation by PKC. A peptide encoding the receptor sequence surrounding these residues inhibited phosphorylation of IR by PKC. In contrast, a mutant peptide in which the Arg and Lys were substituted by neutral amino acids exhibited no inhibitory effects, suggesting that IR phosphorylation by PKC follows direct IR-PKC interaction (23). In the present work we have shown that insulin controls IR association with PKC-α, -δ, and -ζ. In turn, in the NIH-3T3 cells, PKC-α and/or -δ association with IR appears crucial for enabling proper intracellular sorting of the receptor to the insulin degradative route.

EXPERIMENTAL PROCEDURES

Materials—Rabbit polyclonal antibodies toward specific PKC isoforms were purchased from Life Technologies, Inc. Ab50 and J10 receptor antibodies were a generous gift from Drs. D. Accili and P. Gordon (National Institutes of Health, Bethesda, MD). Protein electrophoresis reagents were from Bio-Rad. Western blotting and ECL reagents were from Amersham Corp. Media and sera for cell culture were from Life Technologies Inc. All other reagents were from Sigma.
PKC and Insulin Receptor Intracellular Routing

RESULTS

PKC Co-precipitation with the Insulin Receptor—NIH-3T3 cells expressing human wild-type insulin receptors (3T3_hIR cells) were exposed for 30 min to 100 nM insulin or 1 μM TPA. This treatment increased the ability of plasma membrane preparations from the cells to phosphorylate the Ac-MBP(4–14) substrate for PKC by 2- and 10-fold, respectively (Fig. 1, top panel). Insulin effect was accompanied by a similarly sized increase in phosphorylation of the PKC substrate by specific PKC Abs from basal and insulin- or TPA-stimulated cells (Fig. 2, top panel). At variance, substrate phosphorylation by the IR Ab precipitates from TPA-stimulated cells was increased by only 35 ± 3% (value ± S.D.) above the basal levels. Simultaneous preincubation of the cells with both insulin and TPA did not significantly enhance the effect of insulin alone. Ac-MBP(4–14) phosphorylation by the immunoprecipitates from both basal and insulin or TPA-stimulated cells was blocked by the specific PKC inhibitory peptide PKC(19–36), indicating co-precipitation of PKC with the insulin receptor.

To further investigate the PKC-IR co-precipitation, we immunoblotted the IR Ab precipitates with isoform-specific PKC Abs followed by blotting and detection with IR Abs. Aliquots of the cell lysates were directly immunoblotted with no previous precipitation (total lysates). The experiment shown is representative of four independent experiments.

PKC-α, -δ, and -ζ co-precipitation with the IR exhibited very similar dose responses. Insulin effects were well detectable at 10^{-10} M, half-maximal at 2–5 × 10^{-9}, and reached a plateau at 10^{-7} M. Differing from dose response time courses of the insulin effect on IR-PKC co-precipitation were specific for each PKC isoform. In the case of PKC-α, the insulin effect was maximal after 30 min of exposure, remaining unchanged for up to 2 h, and then vanishing in 16 h (Fig. 4, top panel), while, in the case of PKC-ζ, the effect reached a maximum after 15 min and disappeared by 60 min (Fig. 4, middle panel). For PKC-ζ,
the insulin effect was diphasic, with an early spike (maximum in 5 min), which returned to basal levels in 15 min and was followed by a more sustained increase lasting for up to 16 h (Fig. 4, bottom panel).

Effects of PKC Inhibition and Cellular Depletion on IR Routing—Evidence is available that PKC has an important role in regulating the internalization and degradation of several tyrosine kinase receptors following binding with their specific ligand (6). We therefore addressed the questions whether this is also the case for the IR kinase, and whether specific PKC isoforms are involved in this regulation. To this end, we analyzed insulin-induced IR internalization and intracellular routing following either simultaneous inhibition of PKC-α, -δ, and -ζ activities with staurosporine or cell depletion of TPA-sensitive PKCs (PKC-α and -δ) by a 24-h preincubation with 1 μM TPA. Preincubation of the cells with 6 μM staurosporine before insulin stimulation inhibited PKC activity in total cell lysates by almost 55%. Concomitantly, as revealed by immunoblot studies, the levels of PKC-α, -δ, and -ζ in IR Ab precipitates were reduced by 3-, 4-, and 10-fold, respectively (Fig. 5, top panel), suggesting that PKC activation is necessary for its association with the insulin receptor. Chronic treatment of the cells with TPA decreased PKC activity by >80% and also reduced recovery of PKC-α and -δ in the IR Ab precipitates by 6- and 5-fold, respectively. At variance from the staurosporine however, similar amounts of the PKC-ζ isoform were evidenced in the immunoprecipitates from TPA-treated cells and in the precipitates from untreated cells. Upon entering cells, normal insulin-bound IRs return to the plasma membrane mainly through an intracellular compartment where insulin is detached from the receptor and degraded (26, 28). Small amounts of internalized receptors are also rapidly recycled through a distinct retroendocytotic mechanism (26, 28). Following TPA and staurosporine preincubation, however, the amount of trichloroacetic acid-soluble (degraded) 125I-insulin released by the cells decreased by 66 ± 7 and 32 ± 3%, respectively (Fig. 5, middle panel). These changes were accompanied by 60 ± 5 and 22 ± 4% respective increases in the amount of trichloroacetic acid-precipitable (intact) 125I-insulin released into the medium by TPA- and staurosporine-treated cells, respectively (Fig. 5, bottom panel). No significant change on insulin-induced IR internalization by the cells occurred in either the TPA- or the staurosporine-treated cells (data not shown). Thus, preserved association of the IR with PKC-α and -δ but not PKC-ζ corre-
and time-dependent internalization in response to insulin, superimposable to that of the wild-type IR (data not shown). Preincubation with 100 nM insulin for 0.5, 1, and 16 h also reduced subsequent 125I-insulin binding by 20, 28, and 31% in the 3T3Mut cells and by 19, 25, and 28% in the 3T3 hIR cells, indicating that the mutant receptor undergoes normal insulin-dependent down-regulation as well (Fig. 7, top panel). However, insulin degradation levels were reduced by 3-fold in the 3T3Mut cells as compared with 3T3 hIR cells (Fig. 7, middle panel). Conversely, intact 125I-insulin release into the medium was 3-fold greater in the 3T3Mut than in the 3T3 hIR cells (Fig. 7, bottom panel). Thus, similar to wild-type IR in PKC-depleted cells with IRMut, lack of interaction with PKCs is accompanied by a preferential receptor convey to the cell surface through the retroendocytotic rather than the insulin-degradative route.

**DISCUSSION**

PKC activation in response to insulin has been reported in several tissues and cell types (18, 30). However, neither the molecular mechanisms by which the activation occurs nor the functional role of PKC in the insulin signaling system have been completely elucidated. In the present work, we have shown that, in NIH-3T3 cells expressing human IRs, insulin induces membrane recruitment of PKC-α, -δ, and -ζ, but not of the -ε isoforms. PKC-α, -δ, and -ζ recruitment in response to insulin linearly correlated with their appearance in the IR Ab precipitates. This observation is consistent with a previous report by Liu and Roth (22) in which the recovery of an insulin-stimulated PKC activity in phosphotyrosine Ab precipitates from insulin-stimulated cells was described. While eliciting a 10-fold greater PKC translocation to the plasma membrane, TPA was less effective than insulin in promoting co-precipitation of PKC-α and -δ with the IR and was unable to promote that of PKC-ζ. Neither EGF or platelet-derived growth factor elicited any significant IR-PKC co-precipitation (not shown). It appears therefore that, in NIH-3T3 cells, a specific association of PKC-α, -δ, and -ζ with IR occurs in response to insulin.

We have further observed that, in NIH-3T3 cells, inhibition of PKC-α, -δ, and -ζ activity by staurosporine also inhibited insulin-induced PKC-IR association, suggesting that the insulin-induced activation of these PKC isoforms is necessary to allow their subsequent association with the receptor. Staurosporine pretreatment of the cells also increased the routing of the insulin-receptor complexes through the retroendocytotic pathway, thus shifting the internalized receptors from the degradative to the retroendocytotic compartment. This change in receptor sorting was not accompanied by alterations in insulin-induced receptor internalization or down-regulation, indicating that PKC-IR association is crucial in specifically controlling the
intracellular sorting of the receptor following insulin-dependent internalization. Consistent with these data and with the relevance of a direct PKC-IR interaction for proper sorting of the receptor, the IR$_{QA}$ mutant, which is unable to interact with PKC-α, -δ, and -ζ, but is normally responsive to insulin in term of auto- and substrate phosphorylation, kinase activation, and signaling (23), exhibited identical abnormalities in intracellular cell sorting as the wild-type human IR in staurosporine-signaling (23), exhibited identical abnormalities in intracellular sorting as the IR QA (26).

Similar to IRQA, IRQK is unable to interact with PKC and insulin receptor intracellular routing. Pretreatment of NIH-3T3 cells with TPA before insulin stimulation depleted the cells of PKC-α and -δ, but had no effect on PKC-ζ levels. Accordingly, the levels of PKC-ζ co-precipitating with IRs following insulin stimulation were identical in cells treated with TPA or not, while the levels of PKC-α and -δ were greatly reduced. Nevertheless, in TPA-pretreated cells, we observed a shift of the internalized insulin-IR complexes from the degradative toward the retroendocytotic route that was almost 2-fold more pronounced than that caused by staurosporine. We suggest, therefore, that PKC-α and/or -δ but not PKC-ζ are responsible for PKC control of IR routing in these cells. Based on the findings reported in the present work, PKC-α appears the most likely candidate for this regulatory role. In fact, (i) the time course of insulin-induced IR engagement in the degradative route better correlates with the time course of insulin-dependent PKC-α-IR co-precipitation than with that of PKC-δ-IR co-precipitation, and (ii) chronic exposure of the cells to TPA, to which the IR sorting mechanism is extremely sensitive, determines a 3-fold greater depletion of PKC-α than -δ from the cells. In addition, consistent with this possibility, Seedorf et al. (6) have recently shown that chimeric receptors engineered with the EGF receptor extracellular domain fused to the cytoplasmic domain of several different tyrosine kinase receptors, including IR, form stable complexes with PKC-α upon EGF binding and promote receptor tyrosine kinase internalization and degradation. TPA-sensitive PKCs are known to bind and phosphorylate cytoskeletal proteins such as F-actin (32, 33), talin (34), and other cytoskeleton-associated proteins (35, 36), as well as proteins regulating vesicle formation and trafficking such as dynamin I (37). It is tempting therefore to speculate that, once bound to the IR, PKC-α might control the IR intracellular routing by interacting with specific cytoskeletal elements. Evidence is available that PKCs are required for insulin-induced regulation of gene expression (18, 38), protein synthesis (30), glucose uptake (18, 20, 21), and pyruvate dehydrogenase activity (19). Previous studies have also demonstrated an important role of the PKC system in controlling the IR kinase and signaling in several physiological (39) and pathological (40) conditions. By describing the role of PKC in controlling the IR intracellular sorting, the findings in the present work indicate the existence of an additional step where PKCs may control the function of the insulin receptor and thus insulin action.

Acknowledgments—We are grateful to Drs. E. Consiglio and G. Vecchio for their continuous support and advice during the course of this work, to Drs. P. Gorden and D. Accili (National Institutes of Health, Bethesda, MD) for generously donating anti-insulin receptor antibodies. We also thank Dr. M. Bifulco for helpful discussions and Dr. D. Liguori for the technical help.

REFERENCES

1. Kahn, C. R. (1994) Diabetes 43, 1066–1084
2. White, M. F. (1997) Diabetologia 40, S2-S17
3. Sun J., Grimmins D. L., Myers M. G., Jr., Miralpeix M., and White M. F. (1993) Mol. Cell. Biol. 13, 7418–7428
4. Levy-Toledano, R., Tauxis, M., Blaettler D. H., Gorden, P., and Taylor, S. I. (1994) J. Biol. Chem. 269, 31178–31182
5. Van Horn, D. J., Myers, M. G., Jr., and Backer, J. M. (1994) J. Biol. Chem. 269, 29–32
6. Seedorf, K., Shearman, M., and Ullrich, A. (1995) J. Biol. Chem. 270, 19953–19960
7. Ahn, J., Donner, D. B., and Rosen, O. M. (1993) J. Biol. Chem. 268, 7571–7576
8. Hug, H., and Sarre, T. F. (1993) Biochem. J. 291, 329–342
9. Chabant, C. E., Mischak, H., Watson, J. E., Winkler, B. C., Goodnough, J., Farese, R. V., and Cooper, D. R. (1995) J. Biol. Chem. 270, 13326–13332
10. Newton, A. C. (1997) Curr. Opin. Cell Biol. 9, 161–167
11. Goodnough, J., Mischak, H., Kolch, W., and Ullrich, A. (1995) J. Biol. Chem. 270, 9991–10001
12. Takayama, S., White, M. F., and Kahn, C. R. (1988) J. Biol. Chem. 263, 3440–3447
13. Coughlan, M. P., Pillay, T. S., Tavare, M. J., and Siddle, K. (1994) Biochem. J. 303, 893–899
14. Lewis, R. E., Volle, D. J., and Sandersen, S. D. (1994) J. Biol. Chem. 269, 26259–26266
15. Chin, J. E., Dickens, M., Tavare, M. J., and Roth, R. A. (1993) J. Biol. Chem. 268, 6338–6347
16. Schaubert, C., Carell, K., DePaolo, D., Leitner, W., and Draznin, B. (1999) J. Biol. Chem. 274, 15311–15314
17. Ishizuka, T., Miura, A., Kajita, K., Kanoh, Y., Ishizawa, M., Itaya, S., Kimura, M., Yamada, K., and Yasuda, K. (1997) Diabetes 46, Suppl. 1, S139
18. Farese, R. V., Staender, M. L., Arnold, T., Yu, B., Ishizuka, T., Hoffman J. M., 13201
PKC and Insulin Receptor Intracellular Routing

19. Benelli, C., Caron, M., de Galle, B., Fouque, F., Cherqui, G., and Clot, J. P. (1994) *Biochem. J.* **302**, 271–277
20. Chalfant, C. E., Ohse, S., Konno, Y., Fisher, A. A., Bisauteur, L. D., Watson, J. E., and Cooper, D. R. (1996) *Mol. Endocrinol.* **10**, 1273–1281
21. Liu, F., and Roth, R. A. (1994) *Biochem. Biophys. Res. Commun.* **200**, 1570–1577
22. Miele, C., Formisano, P., Sohn, K. J., Caruso, M., Palumbo, G., Beguinot, L., and Beguinot, F. (1994) *J. Biol. Chem.* **269**, 16242–16246
23. Marshall, S. (1985) *J. Biol. Chem.* **260**, 13524–13531
24. Levy, J. R., and Olefsky, J. M. (1986) *Endocrinology* **119**, 572–579
25. Mendez, R., Kollmorgen, G., White, M. F., and Rhoads, R. E. (1997) *Mol. Cell. Biol.* **17**, 5184–5192
26. Formisano, P., Sohn, K.-J., Miele, C., Di Finizio, B., Petruzziello, A., Riccardi, G., Beguinot, L., and Beguinot, F. (1995) *J. Biol. Chem.* **268**, 5244–5248
27. Zheng, Y., Li, J.-P., Powell, K. A., Sudhof, T. C., and Robinson, P. J. (1994) *J. Biol. Chem.* **269**, 21043–21050
28. Weinstock, R. S., and Messina, J. L. (1988) *Endocrinology* **123**, 366–372
29. Berti, L., Mosthaf, L., Kroder, G., Kellerer, M., Tippmer, S., Mushack, J., Seffer, E., Seedorf, K., and Haring, H. (1994) *J. Biol. Chem.* **269**, 3381–3386
30. King, G. L., Shibata, T., Oliver, J., Inoguchi, T., and Bursell, S. E. (1994) *Annu. Rev. Med.* **45**, 179–188