Diabetologia (1997) 40: 863–866

Diabetologia

Protein kinase C isoforms $\beta 1$ and $\beta 2$ inhibit the tyrosine kinase activity of the insulin receptor

B. Bossenmaier[5], L. Mosthaf[1], H. Mischak[2], A. Ullrich[3], H. U. Häring[4]

[1] Hagedorn Research Institute, Gentofte, Denmark
[2] Hämatologisches Institut, München, Germany
[3] Max Planck Institut für Biochemie, Martinsried, Germany
[4] Eberhard-Karls-Universität, Medizinische Klinik u. Poliklinik, Tübingen, Germany
[5] Boehringer Mannheim, Mannheim, Germany

Summary Downregulation of insulin receptor tyrosine kinase (IRK) activity yields to impaired insulin signalling and contributes to the pathogenesis of cellular insulin resistance. Activation of protein kinase C (PKC) by different agents is associated with an inhibition of IRK activity in various cell types. There is evidence that this effect on IRK activity might be mediated through phosphorylation of specific serine residues of the insulin receptor β-subunit. Neither the domains of the IRK where inhibiting serine phosphorylation occurs nor the PKC isoform responsible for IRK inhibition have been identified. PKC consists of a family of at least 12 isoforms. The aim of the present study was to determine which PKC isoform might be capable of IRK inhibition. The human insulin receptor and the PKC isoforms $\alpha, \beta 1, \beta 2, \gamma, \delta, \varepsilon, \eta, \theta$ and ζ were overexpressed in human embryo kidney fibroblasts (HEK 293 cells) in order to answer this question. PKCs were activated by preincubation with the phorbolester (TPA) (10^{-7} mol/l) following insulin stimulation of the cells. When the IRK was coexpressed with the PKC isoforms $\beta 1$ and $\beta 2$, a 50 ± 15.7 and 45 ± 10.1 % inhibition of tyrosine autophosphorylation of IRK was observed while coexpression with the other isoforms did not significantly modify IRK autophosphorylation. The data suggest that the PKC isoforms $\beta 1$ and $\beta 2$ might be candidates for insulin receptor inhibition. [Diabetologia (1997) 40: 863–866]

Keywords Insulin resistance, insulin receptor, protein kinase C.

The pathogenesis of non-insulin-dependent diabetes mellitus (NIDDM) is determined by abnormalities of insulin secretion and by insulin resistance of the major target tissues. In particular, insulin resistance of the skeletal muscle appears to play a pivotal role in the pathogenesis of the disease [1]. It appears that an impaired signalling capacity of the insulin receptor contributes to the pathogenesis of skeletal muscle insulin resistance as many studies have reported that autophosphorylation or substrate phosphorylation of the insulin receptor tyrosine kinase isolated from diabetic skeletal muscle is reduced (reviewed in [2]). The molecular mechanisms responsible for the reduced activation of the insulin receptor kinase in NIDDM patients have not yet been identified. Experimental conditions such as stimulation of cells by phorbol esters, hyperglycaemia and catecholamines are able to regulate insulin receptor function (reviewed in [2]) and are of particular importance as they suggest that activation of protein serine/threonine kinases might be involved in receptor kinase inhibition. Among the serine kinases which might be involved in insulin receptor inhibition protein kinase C (PKC) is one candidate with potential pathophysiological relevance. PKC plays a key role in transmembrane signal transduction of several hormones, growth factors, and neurotransmitters. In recent years several isoenzymes have been identified and characterized according to

Received: 3 March 1997 and in revised form: 17 April 1997

Corresponding author: Professor H. U. Häring, Eberhard-Karls-Universität, Medizinische Klinik und Poliklinik, Abt IV, Otfried Müller Strasse 10, D-72076 Tübingen, Germany

Abbreviations: NIDDM, Non-insulin-dependent diabetes mellitus; PKC, protein kinase C; IR, insulin receptor; FCS, fetal calf serum; IRK, insulin receptor kinase; PMSF, phenylmethylsulphonyl fluoride; DTT, dithiothreitol; HEK, human embryo kidney cells; TPA.

their molecular and biochemical properties [3]. They represent a family of structurally and functionally related serine/threonine kinases which are derived from multiple genes as well as from alternative splicing of single mRNA transcripts. The isoforms differ in their regulatory domains and in their dependence on Ca^{2+}, as well as in their tissue distribution. Due to these characteristics, PKC isoforms can be subdivided into three major groups: classical, Ca^{2+}-dependent cPKC isoforms ($\alpha, \beta 1, \beta 2, \gamma$), new, Ca^{2+}-independent nPKC isoforms (δ, ε, η, θ), and atypical aPKC isoforms (ζ, λ) [3]. Activation of PKC seems to be associated with intracellular redistribution of the enzyme. In its unstimulated state PKC is localized in the cytosolic fraction, whereas stimulation of the enzyme causes translocation to the plasma membrane [3].

Insulin receptor (IR) inhibition has been observed in several cell systems after phorbolester treatment [4–5]. Recently we obtained evidence that IR inhibition induced by hyperglycaemia is also mediated by PKC [6]. However, it remains unclear which PKC isoform is responsible for the inhibitory modulation of the insulin receptor tyrosine kinase (IRK). The aim of the present study was to investigate which PKC isoform might be a candidate for an IR inhibiting serine kinase. To address this question we performed coexpression studies of the IR with different PKC isoforms in HEK 293 cells.

Materials and methods

Materials. Cell culture reagents and fetal calf serum (FCS) were purchased from Gibco (Eggenstein, Germany); culture dishes were from Greiner (Frickenhausen, Germany). Porcine insulin, aprotinin, phenylmethylsulphonyl fluoride (PMSF), (TPA), Na_3VO_4, Triton X-100, Tween 20 and dithiothreitol (DTT) were from Sigma (Munich, Germany). The reagents for SDS-PAGE and Western blotting were obtained from Roth (Karlsruhe, Germany) and Biorad (Munich, Germany). Nitrocellulose was from Schleicher & Schuell (Dassel, Germany). Visualization of immunocomplexes after Western blotting was performed with the non-radioactive enhanced chemiluminescence system (ECL Amersham, Amersham, UK). Anti-pTyr antibodies (Py20) were from Leinco Technologies, USA. The IR antibody against the C-terminal domain of the β-subunit (αIR) was described earlier [6]. Polyclonal PKC isoform antibodies were purchased from Gibco.

Transient expression of HIR and PKC isoforms in HEK 293 cells. The cDNA for the wild-type IR or the different PKC isoforms ($\alpha, \beta 1, \beta 2, \gamma, \delta, \varepsilon, \eta, \theta$ and ζ) were cloned into a cytomegalovirus promoter-based expression vector and plasmid DNA was prepared using a Qiagen Plasmid Kit. Human embryo kidney (HEK) fibroblast 293 cells (ATCC CRL 1573) were grown in Dulbecco's MEM/Nutrient Mix F12 medium supplemented with 10% FCS. A total of 4 μg plasmid DNA was transfected per semiconfluent 35-mm diameter dish according to the protocol of Chen and Okayama [7]. Cultures were maintained overnight at 37 °C, 3% CO_2. The medium was changed to Dulbecco's MEM/Nutrient Mix F12 medium without FCS containing 2 mmol/l glutamine 20 h before the experiment. Cells were then preincubated with 10^{-7} mol/l TPA for 10 min before stimulation with 10^{-7} mol/l insulin (5 min). Subsequently, cells were lysed in 200 μl lysis buffer (50 μmol/l HEPES pH 7.2, 150 mmol/l NaCl, 1 mmol/l EGTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 100 mmol/l NaF, 10 mmol/l sodium pyrophosphate, 100 μmol/l sodium orthovanadate, 1 mmol/l PMSF, 10 μg/ml aprotinin). The lysates were centrifuged for 10 min at 14000 *g*, 40 μl of the supernatant was taken, 5 × Laemmli buffer added, boiled for 5 min, and separated by 7.5% SDS-PAGE.

Western blotting. After electrophoresis, proteins were transferred to nitrocellulose by electroblotting (transfer buffer: 20 mmol/l NaH_2PO_4 and 20 mmol/l Na_2HPO_4, pH 8.8). After transfer, the nitrocelluloses were blocked with NET buffer (150 mmol/l NaCl, 5 mmol/l EDTA, 50 mmol/l Tris, 0.05% Triton X-100 and 0.25% gelatine, pH 7.4) for 1 h. Subsequently, they were incubated with the first antibodies (αPy20, αCT104 or PKC antibodies in NET buffer) overnight at 4 °C. The nitrocellulose membranes were washed 4 × 10 min with NET buffer before incubating with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG for 1 h at room temperature. Visualization of immunocomplexes was performed by ECL. To remove antibodies before reblotting, the nitrocellulose was incubated in 62.5 mmol/l Tris-HCl, pH 6.8, 2% SDS and 100 mmol/l β-mercaptoethanol for 1 h at 55 °C.

Results

To elucidate which PKC isoforms might be able to modulate the tyrosine kinase activity of the IR we coexpressed in HEK 293 cells the human IR and different PKC isoforms (α, $\beta 1$, $\beta 2$, γ, δ, ε, η, θ and ζ). Transfected 293 cells were incubated with TPA (10^{-7} mol/l) for 10 min to activate PKCs and then stimulated with insulin for 5 min. Whole cells lysates were separated on a 7.5% SDS-PAGE, blotted onto nitrocellulose and immunodetected with anti-phosphotyrosine antibody (Fig. 1, panel A). The expression of the IR or of the PKC isoforms was determined by reprobing nitrocellulose filters with anti-IR (panel B) or anti-PKC antibodies (panel C), respectively. As can be seen in Figure 1, panel A, cells coexpressing the IR and PKC $\beta 1$ or PKC $\beta 2$ show a clear reduction of IR autophosphorylation in comparison to cells overexpressing the IR alone or together with PKC γ, η, θ or ζ. In addition, cells overexpressing the IR together with PKC α show a decreased receptor autophosphorylation when considering the increase in IR in these cells compared to the control. The different intensities of PKC staining of the isoform specific antibodies used in Figure 1 panel C does not reflect different expression levels but different antibody affinities, as verified by use of a pan-PKC antibody (panel D).

Figure 2 summarizes the densitometric quantification of five independent experiments. Tyrosine phosphorylation of the IR expressed alone was set as 100%, and the phosphorylation of the IR coexpressed with a PKC isoform compared. In the presence

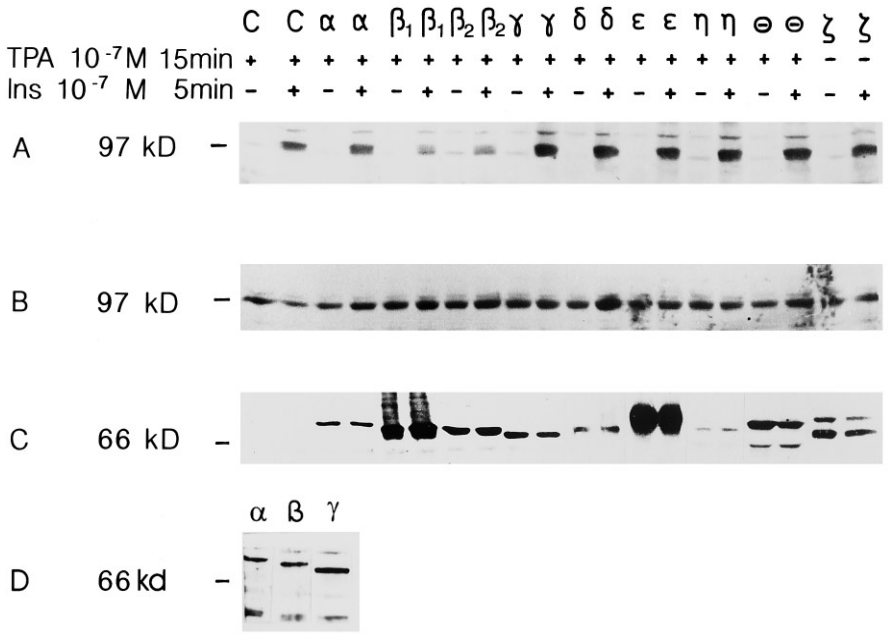

Fig. 1. A–D. Inhibition of IRK by PKC isoforms. HEK 293 cells were transfected with cDNA for the human insulin receptor and different PKC isoforms as indicated. Transfected 293 cells were incubated with 10^{-7} mmol/l TPA for 10 min prior to insulin stimulation. Whole cell lysates were separated on a 7.5% SDS PAGE, blotted onto nitrocellulose and immunodetected with antiphosphotyrosine antibody (panel A). The same nitrocellulose was then reprobed with anti-insulin receptor antibody (panel B), the appropriate anti-PKC antibodies (panel C) and pan-PKC antibody (panel D) as described in the Methods section

of overexpressed PKC $\beta 1$ or PKC $\beta 2$ IR autophosphorylation was reduced by $50 \pm 15.7\%$ ($n = 5$, $p < 0.01$) and $45 \pm 10.0\%$ ($n = 5$, $p < 0.01$), respectively.

These experiments suggest that the PKC isoforms $\beta 1$ and $\beta 2$ are likely to phosphorylate the IR and thus are capable of inhibiting IR autophosphorylation.

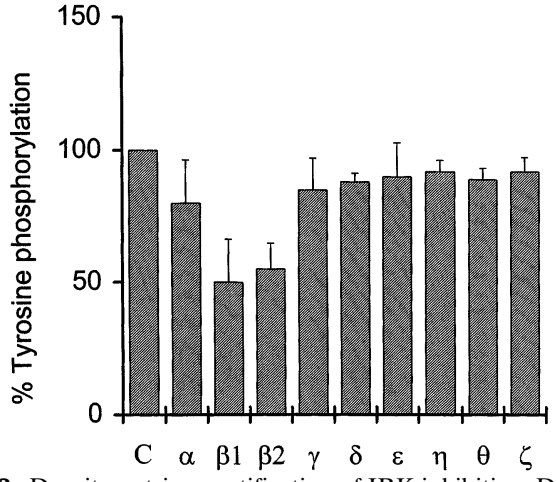

Fig. 2. Densitometric quantification of IRK inhibition. Densitometric data are shown as mean ± SEM of five independent experiments. Tyrosine phosphorylation of the insulin receptor in the presence of insulin was set as 100% and compared with insulin stimulated IRK activity in HEK 293 cells transfected with different PKC isoforms as indicated

Discussion

In an earlier study we demonstrated that in rat-1 fibroblasts hyperglycaemia induced the inhibition of IR autophosphorylation [6]. This effect was mediated by activation and translocation of members of the PKC family to the cell membrane. The data presented in this study suggest that members of the PKC family of protein kinases are capable of interacting directly with the IR in a specific way, probably phosphorylation on serine or threonine residues, that causes inhibition of autophosphorylation.

The interaction of PKC α and $\beta 1$ with the IR was also shown in other cell systems. Chin et al. [8] developed Chinese hamster ovary (CHO) cell lines in which different PKCs, including PKC α and β, were overexpressed. They could show an increase of phosphorylation of the IR in these cell lines but the kinase activity of the IR was significantly impaired only when PKC α was coexpressed while coexpression of PKC $\beta 1$ or γ led to a weak decrease of PI3-kinase activity. However, they did not demonstrate an effect on the autophosphorylation of the IR.

It is likely that different expression levels both of IRs and PKC isoforms might explain the different results. The inhibitory effect of PKC $\beta 1$ and $\beta 2$ on the IR in HEK 293 cells is gradually lost when the cells are transfected with increasing amounts of IR DNA leading to higher receptor levels. This observation suggests that the inhibitory effect observed here

requires an optimal ratio of receptor protein and PKC $\beta 1/\beta 2$ protein in the cell system. It is possible that this optimal ratio of IR and PKC is not obtained in cell lines stably transfected with PKC as only a moderate overexpression of PKC is found in these cells.

Considering the expression levels of different members of the PKC family in insulin target tissues, it is surprising that PKC $\beta 1$ but not θ showed the strongest effect on the IR autophosphorylation since PKC θ is expressed more strongly in skeletal muscle, and in rat soleus muscle it has been found to translocate to the cell membrane upon insulin treatment [9]. On the other hand acute hyperglycaemia causes in rat skeletal muscle a translocation of PKC β [10].

The data available so far provide only very indirect evidence that PKC β might be involved both in insulin downstream signalling as well as inhibitory feedback loops modulating insulin signalling. New experimental approaches involving specific PKC β inhibition might in the future provide more direct evidence for the role of this PKC isoform in the modulation of insulin signalling in target tissues.

Acknowledgement. This study was supported by a grant from the Deutsche Forschungsgemeinschaft to H. U. Häring.

References

1. Beck-Nielsen H, Groop LC (1994) Metabolic and genetic characterization of prediabetic states. J Clin Invest 94: 1714–1721
2. Häring HU, Mehnert H (1993) Pathogenesis of type 2 (non-insulin-dependent) diabetes mellitus: candidates for a signal transmitter defect causing insulin resistance of the skeletal muscle. Diabetologia 36: 176–182
3. Nishizuka Y (1992) Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science 258: 607–614
4. Takayama S, White MF, Lauris V, Kahn CR (1984) Phorbol ester modulate insulin receptor phosphorylation and insulin action in cultured hepatoma cells. Proc Natl Acad Sci USA 81: 7797–7801
5. Häring HU, Kirsch D, Obermaier B, Ermel B, Machicao F (1986) Tumor promoting phorbolesters increase the K_M of the ATP-binding site of the insulin receptor kinase from rat adipocytes. J Biol Chem 261: 3869–3875
6. Berti L, Mosthaf L, Kroder G et al. (1994) Glucose induced translocation of protein kinase C isoforms in rat-1 fibroblasts is paralleled by inhibition of the insulin receptor tyrosine kinase. J Biol Chem 269: 3381–3386
7. Chen C, Okayama H (1987) High-efficiency transformation of mammalian cells by plasmid DNA. Mol Cell Biol 7: 2745–2752
8. Chin JE, Dickens M, Tavare JM, Roth RA (1993) Overexpression of protein kinase C isoenzymes alpha, beta I, gamma, and epsilon in cells overexpressing the insulin receptor. Effects on receptor phosphorylation and signaling. J Biol Chem 268: 6338–6347
9. Yamada K, Avignon A, Standaert ML, Cooper DR, Spencer B, Farese RV (1995) Effects of insulin on the translocation of protein kinase C-theta and other protein kinase C isoforms in rat skeletal muscles. Biochem J 308: 177–180
10. Galante P, Mosthaf L, Kellerer M et al. (1995) Acute hyperglycemia provides an insulin independent inducer for GLUT4 translocation in C_2C_{12} myocyte and rat skeletal muscle. Diabetes 44: 646–651