Endocrine, *vol. 8, no. 2, 161–167, April 1998* 0969–711X/98/8:161–167/$9.75

Translocation of PKC Delta by Insulin in a Rat Hepatoma Cell Line

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The aim of this study was to examine the effects of insulin and phorbol 12-myristate 13-acetate (PMA), an activator of classic and novel PKCs, on the translocation of PKC from cytosol to membrane in H4IIE (H4) rat hepatoma cells. Six PKC isoforms were expressed, including PKC-μ and PKC-λ, identified for the first time in this hepatoma cell line. Insulin induced translocation of PKC-δ from the cytosol to the membrane fraction as early as 15 min and maximally at 60 min with levels returning to that of controls by 180 min. Insulin also decreased levels of PKC-ζ in membranes at 5, 10, 15, and 30 min, but had no effect on cytosol levels. Ten minutes of PMA treatment translocated PKC-δ completely, and 24 h of PMA treatment downregulated PKC-δ. Neither acute nor chronic PMA had any effect on PKC-ζ. These studies establish the ability of both insulin and PMA to activate PKC-δ in H4 cells, and coupled with our previous work demonstrating a diminution of the effect of insulin on gene transcription in PKC downregulated cells, suggest that insulin may exert specific effects, in part, through a PKC-dependent pathway.

Key Words: PKC Delta; insulin; translocation; rat hepatoma cells.

Introduction

Insulin is a potent regulator of cell metabolism and mitogenesis. Although much is known about insulin's effects on cellular metabolism, the intracellular signaling mechanisms that mediate the effects of insulin have not been fully characterized. A general model proposes that insulin, interacting with its cell-surface receptor, initiates phosphorylation/dephosphorylation cascades involving kinases and phosphatases, resulting in the regulation of numerous cellular enzymes as well as transcription factors that in turn modulate gene transcription *(1)*.

Received December 15, 1997; Accepted January 12, 1998.

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The protein kinase C (PKC) family of serine/threonine kinases are involved in peptide hormone signaling in many cell types *(2–4)*. The classic PKCs (cPKC) α, β1, β2, and γ possess in their regulatory domains a C2 region, which binds Ca^{2+}, and a C1 region, which contains two cysteine-rich zinc finger-like motifs that bind diacylglycerol (DAG) and its pharmacological analogs, phorbol esters. Both Ca^{2+} and DAG/phorbol esters are required for their activation *(2–5)*. The novel PKCs (nPKC) δ, ε, η, θ, and μ do not possess the C2 region, but do possess the C1 region, thus requiring DAG/phorbol esters for activation *(2–6)*. The atypical PKCs (aPKC) ζ and λ (also known as τ *[7]*) do not possess the C2 Ca^{2+} binding site, and owing to alterations in their C1 region, are not activated by Ca^{2+} or DAG/phorbol esters *(2–5)*. Because these isozymes vary in their domain structure, regulatory properties, intracellular localization, and tissue distribution, it appears that each is involved in exerting specific biological effects.

The role of PKC in insulin action has not been clearly established. Some studies have demonstrated that insulin induces increased PKC translocation to, and activity in, the membrane fractions of insulin-responsive tissues and cell lines. These support the hypothesis that PKC is involved in some insulin signaling pathways *(8–14)*. Phorbol esters, which activate classic and novel PKCs, provoke many insulin-like responses as well *(15–19)*. In an attempt to understand further insulin's relationship with PKC, we examined more fully the expression and subcellular distribution of the isoforms in H4IIE (H4) hepatoma cells as well as the effects of insulin and phorbol 12-myristate 13-acetate (PMA) on subcellular translocation of PKC, an indicator of PKC activation.

Results

Expression of PKC Isoforms

To characterize more thoroughly the expression of PKC isoforms in H4 cells, we examined the immunoreactivity of 11 isoform-specific antibodies to samples of cytosol and membrane fractions as compared to whole rat brain tissue extract controls and mol-wt standards. Immunoblots revealed the expression of six PKC isoforms in rat H4 cells,

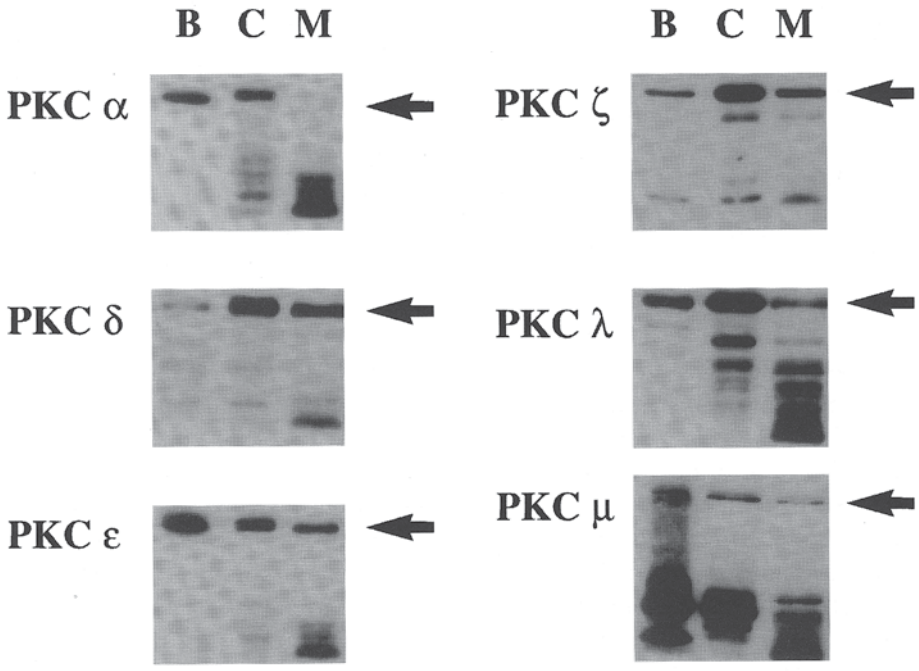

Fig. 1. Identification of PKC isoforms in H4 cells by Western blot analysis. Cells were sonicated and separated into cytosol and membrane protein fractions. Protein samples (50 μg) were separated by SDS-PAGE and transferred to nitrocellulose. Bands were detected using ECL and isoform-specific antibodies. B, rat brain extract; C, cytosol fraction; M, membrane fraction.

two of which are reported here for the first time (Fig. 1). These isoforms were aPKC-λ (also known as a PKC -τ) and nPKC-μ. The cPKC-α with an approximate M_rs of 82 kDa was detected predominantly in the cytosol fraction. The nPKCs δ (78 kDa), ε (90 kDa), and μ (115 kDa), and the aPKCs λ (74 kDa) and ζ (72 kDa) were detected in both the cytosol and membrane fractions. Western blot analysis was also performed for the cPKCs β1, β2, and γ, as well as the nPKCs η and θ. We were unable to detect the expression of these isoforms in 200-μg protein samples in the H4 cells using isoform-specific antibodies, although all five were detected in the rat brain extracts (data not shown).

Effect of Insulin on PKC Translocation

Translocation of PKC in response to agonists is considered to be an indicator of enzyme activation *(2–5)*. Using Western blot analysis with PKC isoform-specific antibodies, we examined the effect of treatment with 5×10^{-8} *M* insulin for 30 s to 180 min on the distribution of PKC isoforms in cytosol and membrane fractions from serum-deprived H4 cells. Stimulation of cells with insulin induced a partial translocation of PKC-δ from cytosol to the membrane (Fig. 2A,B). Measurement of PKC translocation was expressed as a percentage of the controls (untreated cells), comparing control cytosol fractions to treated cytosol fractions and control membrane fractions to treated membrane fractions. Fifteen minutes after insulin treatment, levels of cytosolic PKC-δ decreased slightly to 90 ± 11% (mean ± SEM) of controls with a corresponding increase of 19 ± 20% in membrane levels (data not shown). By 60 min, PKC-δ cytosol levels decreased significantly to 69 ± 4% of controls ($p < 0.05$) with a corresponding increase of 46 ± 24% ($p < 0.05$) in membrane levels (Fig. 2B). Both cytosol and membrane levels began to normalize at 120 min (73 ± 7 and 112 ± 25%, respectively) and returned to that of controls by 180 min (99 ± 11 and 110 ± 17%, respectively).

Acute insulin treatment had no significant effect on levels of cytosol and membrane PKC-α, λ, ε, or μ. Acute insulin treatment did, however, have an effect on levels of PKC-ζ. Although levels of PKC-ζ in cytosol fractions remained relatively constant, there was a significant, but transient decrease to 75 ± 6% ($p < 0.01$) of control values in membrane PKC-ζ at 10 min and decreases of 89 ± 9, 89 ± 10, and 87 ± 9% at 5, 15, and 30 min, respectively (Fig. 3) as well as decreases at 120 min (81 ± 13%, $p < 0.05$) and 180 min (86 ± 13%) (data not shown).

Effect of PMA on PKC Translocation

H4 cells were stimulated for 10 min with PMA, a potent activator of classic and novel PKCs, and analyzed using Western blot analysis. The acute effects of PMA are shown in Fig. 4. As expected, the cPKC-α and the nPKCs δ and ε were completely translocated from the cytosol to membrane fractions by 10 min. The nPKC-μ and aPKCs λ and ζ isoforms were not translocated in response to 10 min of PMA. (Fig. 4).

Twenty-four hours of treatment of H4 cells with PMA resulted in downregulation of several PKC isoforms in both cytosol and membrane fractions. Cytosolic PKC-α was downregulated as was both cytosolic and membrane PKC-δ

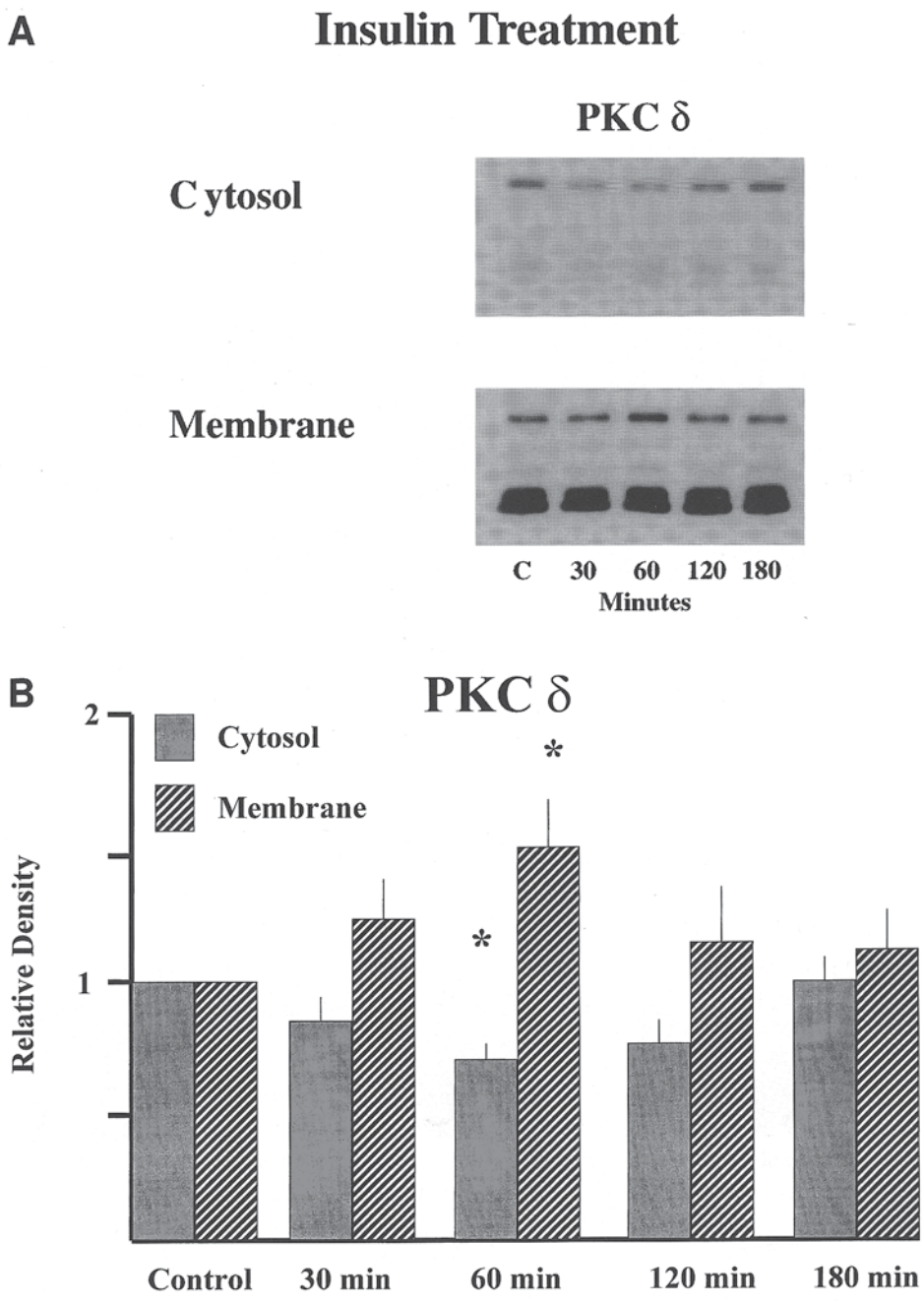

Fig. 2. Time-course showing the effect of insulin on the translocation of PKC-δ. H4 cells were serum-deprived for 24 h and then incubated with $5 \times 10^{-8} M$ insulin for designated times. Cells were harvested, separated into cytosol and membrane fractions, and Western blot analysis was performed as described under Materials and Methods. **(A)** Representative immunoblot of PKC-δ in cytosol and membrane fractions of samples from insulin-treated cells. **(B)** bars represent mean and SE of five separate experiments. The asterisk indicates $p < 0.05$ vs controls.

(Table 1). Cytosol PKC-ε was completely downregulated, whereas membrane PKC-ε was reduced over 70%. Twenty-four hours of treatment of H4 cells resulted in a significant decrease in PKC-μ in the cytosol fraction ($p < 0.01$) with a variable increase in the membrane PKC-μ amount.

Discussion

Evidence from the work of several groups supports the hypothesis that PKC can be activated by insulin in specific target tissues and cell lines *(8–14)*. The liver is an important target tissue for insulin action, and studies examining PKC isoforms in livers of normal and diabetic rats have reported differences in their subcellular localization *(20–23)*. More clinically relevant is a recent report that PKC is increased in the liver of humans with type 2 diabetes mellitus *(22)*. Furthermore, activation of PKC isoforms has been implicated in the development of insulin resistance *(23–26)*, a major characteristic of type 2 diabetes *(27–29)*.

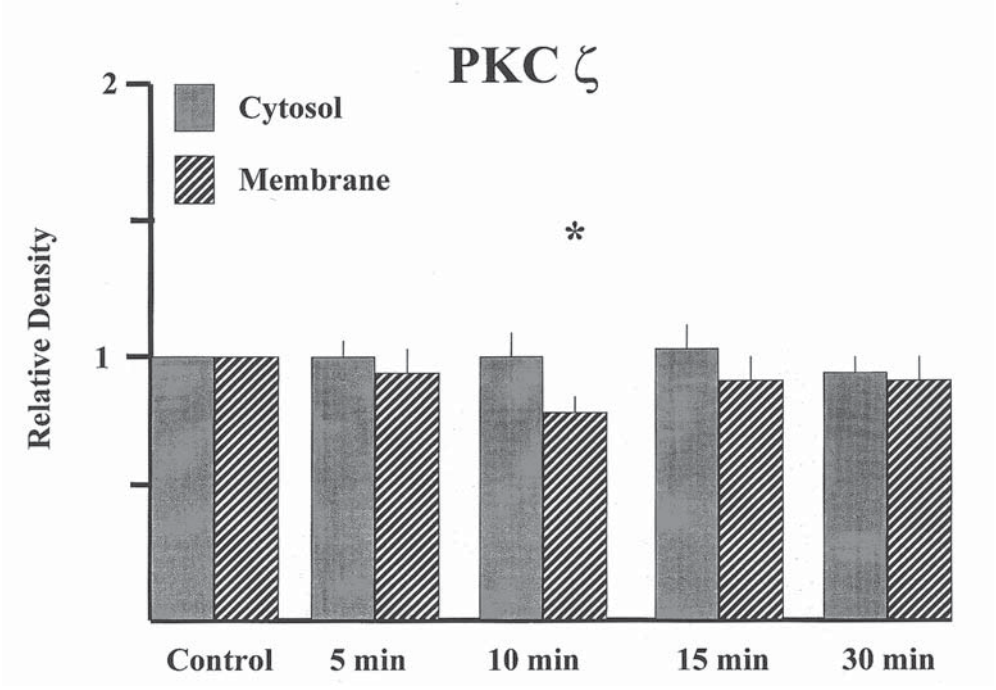

Fig. 3. Time-course showing the effect of insulin on PKC-ζ. H4 cells were serum-deprived for 24 h and then incubated with 5×10^{-8} *M* insulin for designated times. Cells were harvested, separated into cytosol and membrane fractions, and Western blot analysis was performed as described under Materials and Methods. Bars represent mean and SE of five separate experiments. The asterisk indicates $p < 0.01$ vs controls.

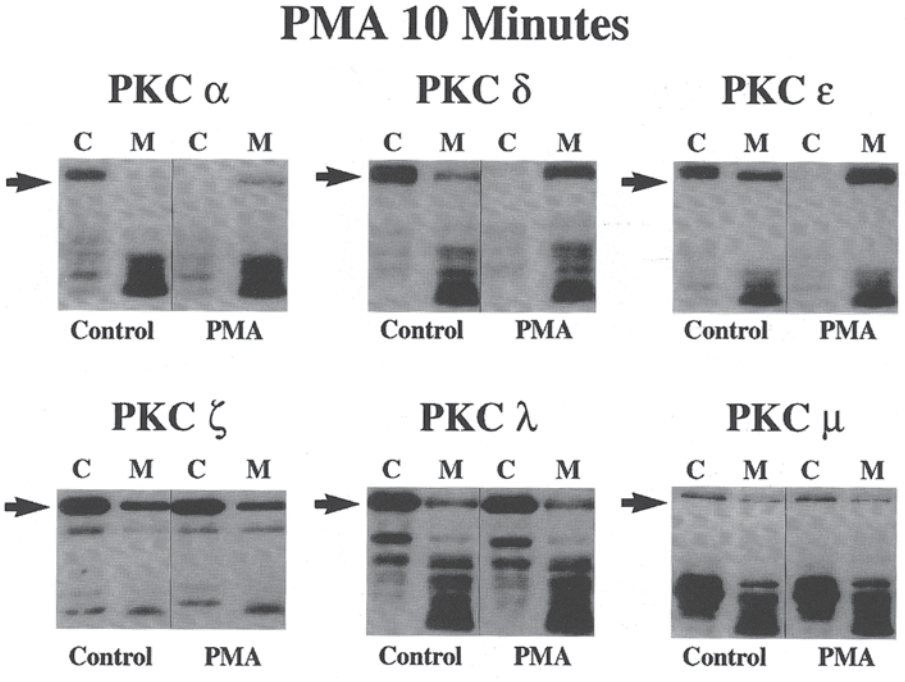

Fig. 4. Effect of acute PMA on the translocation of PKC isoforms in H4 cells. H4 cells were serum-deprived for 24 h and then incubated with 1.6 μ*M* PMA for 10 min. Cells were harvested, separated into cytosol and membrane fractions, and Western blot analysis was performed as described in Materials and Methods. For each isoform, the first two lanes are the cytosol fraction, C; and the membrane fraction, M, from untreated control cells. The third and fourth lanes are cytosol and membrane fractions from PMA-treated cells. Western blots are representative of four separate experiments.

Cell-culture models are useful for elucidating the signaling pathways involved in mediating the effects of insulin. H4 cells were used in these studies because they have retained many liver-specific characteristics and respond to many of the effects of insulin similarly to rat liver *(30)*. Numerous groups have studied the distinct characteristics,

Table 1
The Effects of 24 h PMA Treatment on PKC Isoforms in H4 Cells[a]

	Control, untreated		24 h PMA, % of control		*p* value[b]	
PKC isoform	Cytosol	Membrane	Cytosol	Membrane	Cytosol	Membrane
α (*n* = 3)	100	0	0	0	<0.02	—
δ (*n* = 3)	100	100	0	5	<0.01	NS[c]
ε (*n* = 3)	100	100	0	28	<0.01	<0.03
ζ (*n* = 2)	100	100	80	116	NS[c]	NS[c]
μ (*n* = 2)	100	100	14	192	<0.01	NS[c]

[a]H4 cells were treated with 1.6 μ*M* PMA for 24 h, harvested, separated into cytosol and membrane fractions, and Western blot analyses performed and quantitated as described under Materials and Methods. PMA-treated cells were compared to untreated cells in each individual experiment.

[b]Control untreated vs 24 h PMA treated; paired Student's *t*-test.

[c]NS = not significant.

subcellular localization, and tissue-specific expression of the PKC isoforms as well as their response to a variety of agonists. Many of these data support the hypothesis that each isoform is involved in regulating specific cellular functions *(2–4)*.

We report here that acute insulin treatment of H4 cells induced a small, but significant translocation of PKC-δ from the cytosol to the membrane fraction. The translocation was phasic, beginning as early as 15 min after insulin treatment with maximal translocation at 60 min and a return to control levels by 180 min.

A number of studies suggest a role for PKC-δ in a variety of cellular functions, including regulation of proliferation and differentiation *(31–36)* and the transcription of phorbol ester-inducible genes through both AP-1 and non-AP-1 sequences. *(37)*. PKC-δ has also been shown to be activated by its association with phosphatidylinositol 3-kinase following stimulation by granulocyte macrophage colony-stimulating factor (GM-CSF), an agonist mediated by a tyrosine kinase receptor *(31)*. In the present study, the translocation of PKC-δ by insulin followed a similar time-course to the stimulation of transcription of several early-response genes reported previously, which are often induced 15 min after treatment, but return to control levels by 120 min *(15,16,38–40)*. Furthermore, the phorbol ester, PMA, induced a rapid and complete translocation of PKC-δ, which might correspond to the stronger and more prolonged response of the early-response genes to PMA treatment reported in our earlier studies *(15,16,40)*. The greater efficacy of PMA as compared to insulin would be expected. PMA has potent pharmacological effects, is not readily metabolized by cells, and can prolong the activation of PKCs. Twenty-four hours of PMA treatment caused a complete downregulation of PKC-δ in both fractions. These experiments demonstrating the ability of insulin and PMA to activate PKC-δ in H4 cells, coupled with our previous work on the diminution of the insulin effect in PKC downregulated cells, suggest that insulin is acting at least in part through a PKC-dependent pathway to regulate transcription of at least some genes. This hypothesis is supported further by the fact that chronic PMA treatment downregulated all of the classic and novel PKCs, perhaps impairing the ability of acute insulin to induce gene transcription fully *(41)*.

In earlier studies, insulin was shown to stimulate DNA synthesis, cell division, and transcription of several early-response genes, including c-*fos*, gene 33, β-actin, and γ-actin in H4 cells *(15,16,38,39,42–44)*. PMA mimics some of these effects of insulin *(15,16,38–40)*. Previously, we demonstrated that in H4 cells made deficient in PKC by pretreatment with PMA for 24 h, cytosolic and membrane PKC activity was reduced by 20–45% *(40)*. By Western analysis, the translocation pattern of PKC-β was almost identical to that of total cellular PKC activity *(40)*. In the present work, using the more recently available PKC-β antibodies, we were unable to observe any discernible PKC-β isoform, agreeing with a recent publication *(45)*. This suggests that the older antibody was crossreacting with PKC isoforms shown to be expressed in H4 cells (present study) and not PKC-β as originally described.

In this study, PKC-ζ responded to insulin treatment as well. Like PKC-δ, PKC-ζ was constitutively expressed in both the cytosol and membrane. However, unlike PKC-δ, PKC-ζ was not insulin-activated as measured by translocation. Instead, the activated form in the membrane decreased significantly at 10 min. Association of PKC with the membrane of cells induces activation and triggers the degradation of the protein kinase by proteases. This finding suggests that insulin may be either inhibiting PKC-ζ activation or reducing the pool of activated PKC-ζ by enhancing degradation. A number of studies support the involvement of PKC-ζ, like PKC-δ, in the regulation of cell growth *(46–49)*. Perhaps insulin modifies the balance of activation and degradation of PKC-ζ and PKC-δ to regulate properly the growth of H4 cells.

Of the 11 currently identified mammalian isoforms, H4 cells express 6, 4 of which have been previously reported, but only in whole cellular extracts *(43)*. These include PKCs α, δ, ε, and ζ. Using subcellular fractionation, PKC-α was localized almost exclusively in the cytosolic fraction, whereas PKCs δ, ε, and ζ were detected in both the cytosol and membrane fractions. The isoforms aPKC-λ and nPKC-μ, identified for the first time in H4 cells, were also expressed in both fractions. As has been observed in many cell lines and tissues *(4)*, H4 cells possess isoforms from all three classes of PKCs. The fact that five of the expressed PKC isoforms were constitutively expressed in the membrane fraction, presumably in their activated forms, suggests that they may be necessary for the regulation of cellular housekeeping functions.

PMA activates the classic and novel PKCs. In this study PKCs α, δ, and ε responded to acute PMA treatment as expected by translocating completely from the cytosol to the membrane by 10 min. As expected, 24 h of treatment with PMA downregulated PKCs α, δ, and ε and had no effect on a PKC-ζ. Unexpectedly, our data show that PKC-μ was not translocated by acute PMA treatment, but was partially decreased in the cytosol fraction after 24 h of PMA treatment. A recent study on the activation of PKC-μ demonstrated that this isoform avidly binds phorbol esters *(6)*. It is unclear why we did not observe a stronger PMA effect.

In summary, H4 hepatoma cells expressed six of the known mammalian PKC isoforms that differ in their classifications, quantity, and subcellular localization. Acute insulin treatment affected two of the expressed isoforms, PKC-δ and PKC-ζ, but in different ways. PKC-δ was translocated and thus activated by insulin, whereas the membrane-bound PKC-ζ was depleted. Both PKC responses were transitory. The phorbol ester, PMA, rapidly and completely translocated PKC-δ, but had no effect on PKC-ζ. Chronic treatment with PMA resulted in the downregulation of PKCs α, δ, and ε, and a partial downregulation of μ in the cytosol fraction. Previous studies conducted in our laboratory on the regulation of insulin-responsive genes provided evidence that insulin acts through different pathways to exert its effects on gene transcription, with a possible involvement of PKC. These results further support the involvement of PKC-dependent pathways in insulin signal transduction in H4 hepatoma cells by demonstrating a direct effect of insulin on specific PKC isoforms. The network of intracellular signaling pathways used by insulin is complex, however, and may involve crosstalk and interaction to exert its varied effects. Any association with insulin-induced changes on PKC isoform distribution and gene expression is preliminary and speculative. Further studies must be conducted to determine if PKC-δ is involved directly in the induction of early-response genes by insulin.

Materials and Methods

Materials

Swim's 77 medium and all sera were purchased from Gibco (Grand Island, NY), and porcine insulin and PMA from Sigma (St. Louis, MO). Protein analysis was performed using the Bio-Rad Protein Assay (Hercules, CA). For Western blotting, the mini-electrophoresis system from Bio-Rad was used. Immobilon-NC was obtained from Millipore (Bedford, MA), the ECL detection kit from Amersham (Arlington Heights, IL), monoclonal antibodies (MAbs) to PKC isozymes α, β, γ, δ, ε, ζ, θ, λ, and μ, from Transduction Laboratories (Lexington, KY), polyclonal antibody to PKC isozyme η from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and polyclonal antibodies to PKC isozyme β1 and β2 from Gibco. Whole rat brain extracts were provided by Mary Standaert.

Cell Culture

Rat H4IIE hepatoma cells (American Type Tissue Culture Collection, Rockville, MD) were grown in 150-mm tissue-culture dishes in Swim's medium supplemented with 100 μg/mL gentamicin, 4 m*M* glutamine, 0.05 m*M* cysteine, 26.2 m*M* sodium bicarbonate, 1.8 m*M* calcium chloride, 2% fetal bovine serum, 2% calf's serum, and 2% horse serum in a 5% CO_2 incubator at 85% humidity. Serum was removed 18–20 h prior to experiments when cells were approx 55% confluent. Cells were treated with 5×10^{-8} *M* insulin or 1.6 μ*M* PMA for designated times.

Subcellular Fractionation

After treatment, cells were washed 3× with cold Dulbecco's salt solution, harvested in 350 μL cold PKC buffer (20 m*M* Tris-HCl, pH 7.5, 0.25 *M* sucrose, 5 m*M* EGTA, pH 8.0, 20 m*M* β-mercaptoethanol, 20 μg/mL leupeptin, 0.1 m*M* PMSF, 4 μg/mL aprotinin) and sonicated for three 10-s pulses on ice. The resulting homogenate was centrifuged at 100,000*g* for 30 min to separate the soluble (cytosol) fraction from the particulate (membrane) fraction. The pellet was then resuspended in cold PKC buffer containing 1% Triton-X and 2 m*M* EDTA, pH 8.0, sonicated on ice for three 10-s pulses, and centrifuged again at 100,000*g* for 30 min. The supernatant was considered the soluble membrane fraction. Samples were diluted with 5× sample buffer and frozen at –80°C. Protein concentrations were determined using the Bio-Rad Protein Assay.

Western Blotting

Protein samples were boiled for 2 min, and 50 μg of either the cytosol or membrane fractions were applied to 8% SDS-polyacrylamide gels and resolved at 50 mA/gel for approx 50 min. The gels were equilibrated for 30 min in transfer buffer (0.025 *M* Tris, 0.192 *M* glycine, and 20% methanol) and transferred onto Immobilon-NC using a Hoefer "Semiphor" transfer unit (0.8 mA/cm^2 of gel). Filters were blocked at 4°C overnight in 5% BSA in TBS-

T buffer (20 m*M* Tris-base, 137 m*M* NaCl, 0.1% Tween-20) and incubated with the appropriate PKC isozyme antibody (using recommended dilutions) in blocking buffer for 60 min at room temperature. The filters were washed once for 15 min and twice for 5 min in TBS-T buffer, and subsequently incubated in sheep antimouse IgG conjugated with horseradish peroxidase (1:10,000) for 60 min. The above washing procedure was repeated and bands detected using ECL. The films were scanned on an HP 4C optical scanner, and band analysis was performed on a Macintosh Quadra 800 computer using the public domain NIH Image program (written by Wayne Rasband at the US National Institutes of Health and available from the Internet by anonymous ftp from zippy.nimh.nih.gov or on floppy disk from NTIS, 5285 Port Royal Rd., Springfield, VA 22161). Statistical analysis was performed using a paired Student's *t*-test.

Acknowledgments

We thank Mary Standaert for providing rat brain extracts and for her helpful discussions. This work was supported by the Department of Veterans Affairs Research Funds (R. S. W.).

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