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ZETA PKC IN RAT PREADIPOCYTES : MODULATION BY INSULIN AND SERUM MITOGENIC FACTORS AND POSSIBLE ROLE IN ADIPOGENESIS

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Summary : Several PKC isoforms belonging to the three PKCs'subfamilies cPKC α and β isoforms, nPKC ε isoform and aPKC ζ isoform were detected by Western blot in rat preadipocytes. ζ PKC which appears involved in proliferation and differentiation of some cellular types was shown to display variations according to the preadipocyte anatomical origin and stage of differentiation. A rapid increase in ζ PKC in the cytosolic compartment and translocation into the nucleus were induced by mitogenic factors in proliferating preadipocytes and by insulin in differentiating preadipocytes. These findings suggest that ζ PKC could be involved i) in the post-receptor signaling pathway of serum mitogenic factors and insulin in preadipocytes, and ii) in the mechanisms underlying the variations in the proliferating and differentiating capacities of preadipocytes according to their anatomical localization.

Protein kinase C (PKC) is a family of about twelve different isoforms classified into three groups : the conventional PKC (cPKC) (α, β and γ) which are calcium-dependent, the calcium-independent novel PKC (nPKC) (δ, ε, η and θ) and the atypical PKC (aPKC) (ζ, μ, and ι) which is also calcium-independent and lacks the phorbol ester/diacyl glycerol binding domain (1,2). These isoforms are expressed in a tissue-specific manner and play a regulatory role on cell growth and differentiation (3). For example, β PKC and ζ PKC are necessary for mitogenic signal in erythroleukemia cells (4) and fibroblasts (5) and for differentiation of PC 12 and leukemia cells (6-8).

Obviously, PKC activation on the whole intervenes in the process of proliferation and differentiation of selected adipose cell lines (9-10) and rat preadipocytes as well (11).However, the role played by the different PKC isoforms in these processes is unsettled. Growth hormone exerts a mitogenic and adipogenic role on Ob 17 mouse preadipocyte cells through stimulation of PKC-dependent signaling pathways (12). In 3T3-L1 preadipocytes, the only PKC isoforms so far characterized were the α and β isoforms (13). In addition, βPKC has been suggested to play a role in the 3T3-L1 differentiation process (14).

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In mature adipocytes, several PKC isoforms were characterized (α, β, γ, δ, ε and ζ) (15-17) . These isoforms (except for δ) are translocated from the cytosol to the plasma membrane by insulin (16, 17) but their physiological functions remain unknown.

Marked site-related differences have been found in the metabolic specificities of adipose tissue (18,19). Recently, we have also shown that the PKC system varies according to the anatomical origin of the adipose tissue since PKC content is lower in subcutaneous than in deep intraabdominal adipocytes (20,21). Adipose tissue growth is also influenced by its localization (22,23). For example, perirenal fat deposits contained more replicating and differentiating adipose precursor cells than epididymal adipose tissue (22,23). Moreover, adipose precursor cells from subcutaneous territories express less extensive maturation than those from epididymal fat deposits (24).

The purpose of this study was first to determine whether some PKC isoforms thought to be involved in cell growth and differentiation (α, β, ε and ζ) are present or not in rat preadipocytes during adipogenesis since, until now, only two isoforms (α and β) have been characterized in 3T3-L1 preadipocytes. Second, because preadipocyte growth and differentiation varies according to fat localization, we have focused our attention on the expression of the ζ PKC isoform studied in preadipocytes from different anatomical origins and stages of culture. Third, the possible involvement of ζ PKC in the signaling pathways of mitogenic growth factors and insulin has also been examined.

MATERIALS AND METHODS

Dulbecco's Modified Eagle medium (DMEM), DMEM-Ham's F12 (50 : 50 mix), fetal bovine serum (FBS) and the antisera and synthetic peptides specific for a, β and PKC were obtained from Gibco BRL (Grand Island, NY, USA), ECL Western blotting protocols were purchased from the Radiochemical centre (Amersham, Bucks, UK).
The antiserum and the synthetic peptide specific for the ε PKC were obtained from K. Ways (25).All the other chemicals were of reagent grade.

Cell culture

Cell preparation and culture were performed as described in (26). Briefly, male Sprague-Dawley rats (125-150g) were killed by decapitation and femoral subcutaneous (SC) and epididymal fat samples were removed under sterile conditions. Preadipocytes were obtained by collagenase digestion. Cells were plated and after 12 h, washed with DMEM and fed with DMEM-8% FBS. Medium was changed every other day. At confluence (2-3 days post plating), cells were harvested or allowed to differentiate in DMEM-Ham's F12 containing 5 μg/ml insulin, 10 μg/ml transferrin, 2.5 μg/ml sodium ascorbate and 200 pM T3 (ITT medium) (26).

Subcellular fractionation procedure

Subcellular fractions of preadipocytes and adipocytes were prepared as described in (21). Isolated nuclei were prepared as described in (27). Briefly, cells were resuspended in cold buffer A (50 mM Tris pH 7.4, 10% glycerol, 0.1% Triton X-100, 10 mM K Cl) containing 1 M sucrose. Then, cells were homogeneized on ice in a glass Potter fitted with a teflon pestle and carefully layered on cold buffer A containing 1.4 M sucrose. After centrifugation at 2,500 g for 20 min at 4°C, the resulting nuclear pellet was washed and resuspended in cold buffer containing 50 mM Tris pH 8.0, 120 mM NaCl, 1% Nonidet P40, 0.5% desoxycholate, 0.1% SDS and. The contamination of nuclei was evaluated by measuring the activity of marker enzymes as described in (28) and was below 2% for cytosol and plasma membrane.

Western blot analysis

Equal amounts (10-50 μg protein) of preadipocyte cytosolic, nuclear and membrane fractions were subjected to SDS-PAGE (10% acrylamide). Western blot analysis was performed as previously described (20,21). Specificity of the immunoreactive PKC

isoforms was verified -1) by loss of immunoreactivity of samples when incubated with the antiserum in the presence of the peptide used to prepare the antiserum as recommanded by K. Ways (25) and -2) by comparison with isoforms present in brain fractions.

Glycerol 3-phosphate deshydrogenase (GPDH) assay

Cell differentiation was followed by measuring the GPDH activity according to the method of Wise and Green (29). This activity was : 34 ± 5 and 47 ± 11 mU/10^5 cells for femoral SC and epididymal cells, respectively.

Other determinations

Cell number, cell viability and protein concentration were assessed as previously described (30). All results are expressed as the means ± SEM from at least three individual experiments. Comparisons between groups were made using Student's *t* test.

RESULTS

1. CHARACTERIZATION OF PKC ISOFORMS IN RAT PREADIPOCYTES

Western blot analyses of cytosolic and membrane fractions revealed the following : antiserum specific for α PKC detected immunoreactive species of about 67 kDa with a mostly cytosolic distribution (about 80 %) in confluent and differentiated preadipocytes and adipocytes. Furthermore, the membrane content of α PKC tended to decrease during cell differentiation (Figure 1A). As shown in Figure 1B, immunoreactive bands of 55 kDa were detected with antiserum specific for β PKC. This isoform was distributed mostly in cytosolic (about 75%) for confluent and differentiated preadipocytes whereas for adipocytes, this isoform was equally distributed between the

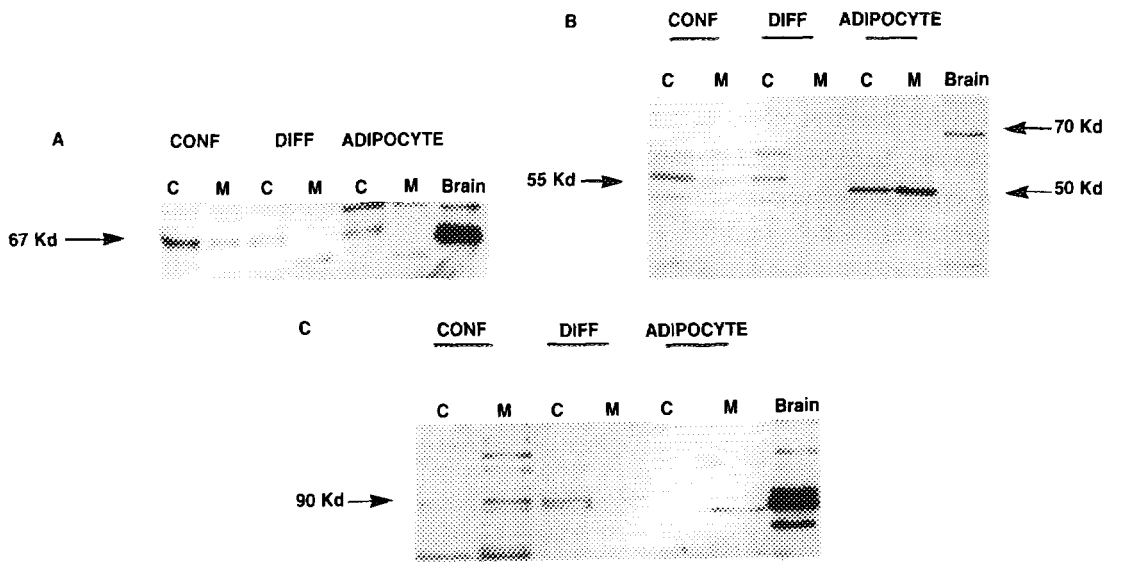

FIGURE 1.
IMMUNOREACTIVE PKC ISOFORMS IN RAT PREADIPOCYTES AND ADIPOCYTES.
Western blot analyses were performed on cytosolic (C) and membrane (M) fractions (30-50 μg protein) from rat preadipocytes at confluent (CONF) and differentiating (DIFF) states and on adipocytes. Brain extract (15 μg protein) was used as positive control.Fractions were probed for α PKC (A) β PKC (B) and ε PKC (C).

cytosolic and membrane fractions. Yet, no important variations could be seen during differentiation of preadipocytes. It should be noted that the molecular weight of this isoform is lower in preadipocytes and adipocytes than in brain. Such a difference has been also reported for the ζ PKC isoform (31). Antiserum specific for ε PKC detected one immunoreactive band of approximately 90 kDa (Figure 1B) in both fractions of preadipocytes and mature adipocytes. However, no important variations of the ε PKC level in cytosolic and membrane fractions could be observed according to the stage of culture of preadipocytes.

2. ζ PKC STATUS

ζ PKC status was next studied in both cytosolic and membrane fractions at different stages of differentiation and according to the anatomical origin of the preadipocytes. The corresponding fractions of mature adipocytes were also included in the experiments. In both subcellular fractions, an immunoreactive band of about 70 kDa was detected by the antiserum specific for ζ PKC (Figure 2). This isoform was equally distributed in the cytosolic and membrane fractions at confluence, but during differentiation, the cytosolic content tended to increase to the detriment of the membrane fraction (about -40%, $p<0.05$) particularly in epididymal cells (71 ± 9% in cytosol, $p<0.05$).

Considering fat localization, immunoreactive amounts of ζ PKC were higher in membrane fractions from epididymal than SC preadipocytes (about + 200%, $p<0.05$) whatever the stage of culture (Figure 2). However, no important variations in the cytosolic content of ζ PKC could be observed according to the anatomical origin of preadipocytes. This finding was strengthened by the fact that the cytosolic protein content was equal in confluent and differentiated preadipocytes whatever their anatomical origin (data not shown). Furthermore, in each subcellular fraction, no difference could be noted in ζ PKC content between SC and epididymal mature adipocytes. Experiments performed in parallel also revealed immunoreactive ζ PKC in both cytosolic and membrane fractions of confluent and differentiated 3T3-F442A cells (unpublished results).

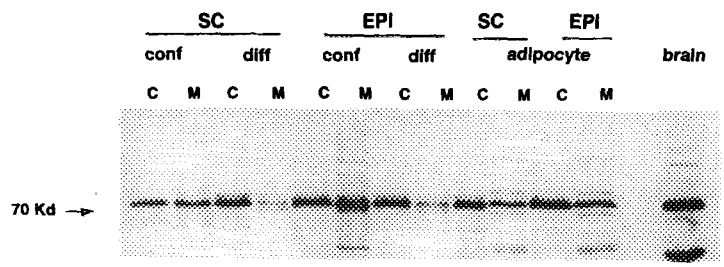

FIGURE 2.
IMMUNOREACTIVE ζ PKC IN RAT PREADIPOCYTES AND ADIPOCYTES.
Western blot analyses were performed on cytosolic (C) and membrane (M) fractions (15 μg protein) from rat confluent (CONF) and differentiating (DIFF) preadipocytes and adipocytes obtained from femoral subcutaneous (SC) and epididymal (EPI) fat deposits. Brain extract (15 μg) was used as positive control.

3. INFLUENCE OF MITOGENS ON ζ PKC SUBCELLULAR DISTRIBUTION

ζ PKC subcellular distribution was studied in response to serum mitogenic factors in proliferating preadipocytes and in response to insulin in differentiating preadipocytes. In proliferating preadipocytes, a rapid increase in cytosolic ζ PKC was observed (about + 150% at 20 min) after serum induction (Figure 3A) with a concomittant decrease in membrane fractions (about -40% at 10 min). It is noteworthy that the same changes occurred in differentiating cells after 20 min-exposure to insulin (10 nM) (Figure 3B). It should be also noted from Figures 3A and B that ζ PKC can be clearly seen as a doublet of proteins in both subcellular fractions of confluent and differentiated cells. One possible explanation for this doublet is that it represents alternatively spliced or post-translationally modified forms of PKC (31). Interestingly, a larger size species cross-reacting with the antiserum specific for ζ PKC was present especially in cytosol after exposition to serum and in membrane after insulin induction. Further experiments are necessary to establish the nature of this additional band.

4. ζ PKC TRANSLOCATION INTO NUCLEUS

Next, nuclear proteins from proliferating and differentiating cells exposed or not to either serum or insulin were analyzed for ζ PKC isoform. Figure 4 shows that nuclei

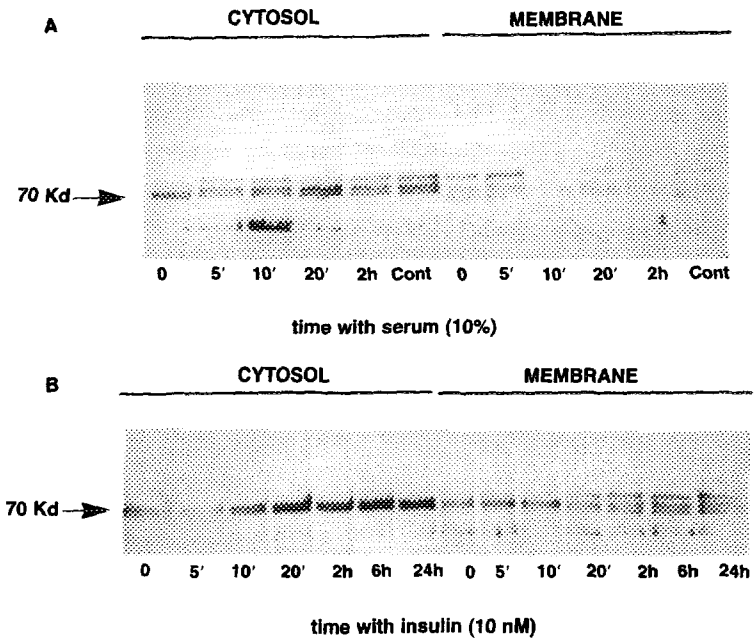

FIGURE 3.
TIME-DEPENDENT EFFECTS OF SERUM (A) AND INSULIN (B) ON SUBCELLULAR REDISTRIBUTION OF ζ PKC IN RAT PREADIPOCYTES.
Proliferating (A) and differentiating (B) preadipocytes were maintained in serum- or insulin-free medium for 18 h and then treated with serum (10%) (A) or insulin (10 nM) (B) for the indicated time periods. Cytosolic (left) and membrane (right) fraction changes are shown from one representative experiment repeated four times with identical results. Controls (CONT) represent the fractions of non-deprived preadipocytes.

from both serum-deprived proliferating and insulin-deprived differentiating cells contained ζ PKC. Furthermore, nuclei from preadipocytes exposed to serum or insulin displayed a 50% increase in immunoreactivity for ζ PKC as early as after 15min-exposure to the effectors. Similar effects of insulin on ζ PKC translocation could also be observed in nuclei of mature fat cells (data not shown).

DISCUSSION

The present study is the first to demonstrate the presence of α and β PKC isoforms in rat preadipocytes, a situation which is similar to that recently reported by Ueda et al. in the 3T3-L1 cell line (14,15). In addition and also for the first time we reported the presence of ε and ζ PKC, two isoforms so far unidentified in the 3T3-L1 cell line. Moreover, the ζ PKC isoform appears to be present in a relatively high amount in preadipocytes.

Adipogenesis seems to depend on the development of the insulin signaling pathway rather than on the PKC pathway (32-34). In fact, insulin receptors and IRS-1 (Insulin Receptor substrate 1) increase during preadipocyte differentiation (32, 33). On the other hand, PKC activity on the whole decreases during differentiation of 3T3-L1 preadipocytes (34) in spite of the possible involvement of the β PKC isoform in 3T3-L1 adipogenesis (15). In preliminar experiments and by using phorbol ester binding techniques, we also observed a 50% decrease of PKC content (unpublished results), but were unable to see any variation of β PKC during differentiation.

Contrasting with β PKC, ζ PKC content showed variations during rat preadipocyte differentiation with a net decrease in membrane fractions when cells are differentiated Furthermore, at confluence, ζ PKC content was higher in epididymal than in SC preadipocytes suggesting that this isoform could be involved in the mechanisms underlying the differences in proliferating capacities between preadipocytes from superficial and deep-intraabdominal fat deposits (24).

A role for ζ PKC in preadipocyte proliferation and differentiation is strongly suggested by our data dealing with the effects of serum growth factors on proliferating cells on one hand, and the effects of the adipogenic hormone insulin on differentiating preadipocytes on the other one. As a matter of fact, our experiments showed in both

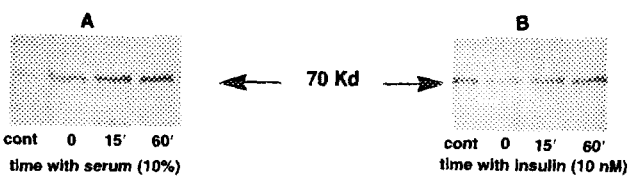

FIGURE 4.
EFFECTS OF SERUM AND INSULIN ON ζ PKC TRANSLOCATION TO NUCLEI OF PREADIPOCYTES.
Proliferating (A) and differentiating (B) preadipocytes were maintained in serum- or insulin-free medium for 18 h and then treated with serum (10%) (A) or insulin (10 nM) (B) for the indicated periods. Nuclei (10^7 cells) were isolated as described in Materials and Methods and probed with anti- ζ PKC antibody. The data presented are from one representative experiment among four.

cases a rapid increase of ζ PKC content in the cytosol with a concomittant decrease in the membrane fractions after exposure to either serum or insulin. Recently, the same phenomenom has been observed in PC12 cells after exposure to NGF which also induces differentiation of these cells (6). In PC 12 cells (6), like in rat preadipocytes (this study), the kinetics of these effects are too rapid to be representative of increased transcription of the ζ PKC gene. Thus, what we observed after activation by serum or insulin is most likely representative of subcellular redistribution of ζ PKC. Moreover, these findings suggest that ζ PKC translocation to the membrane is not absolutely required for the activation of this isoform. It must be noted however that PIP2, the product of the membranous enzyme PI-3 kinase, which is also a target for insulin, induces ζPKC activation in vitro (35).

Our present study also show the presence of ζ PKC in nuclei of unstimulated preadipocytes. Moreover, when cells were stimulated by growth factors or insulin, we found an additional and rapid increase in ζ PKC nuclear content. Since, several nuclear transport signal sequences are present in ζ PKC (31), our data suggest that upon cell activation, ζ PKC may directly phosphorylate structural and/or regulatory nuclear proteins (31).

Stimulation of PKC on the whole has been proposed as one of the post-receptor signaling pathway of insulin and, in mature adipocyte, the PKC isoform involved has been suggested to be β PKC (36). Another possibility is that insulin activates other PKC isoforms. In rat hepatoma cells, it was indeed reported that insulin still stimulates c-fos protooncogene transcription even when PKC is down-regulated by TPA(37). Our results showing after insulin stimulation a subcellular redistribution and nucleus translocation of ζ PKC which is insensitive to TPA, argues in favor of a central role for ζ PKC in insulin signaling pathway, at least, in adipose precursor cells. ζ PKC could thus be integrated in the mitogenic effect of insulin in preadipocytes all the more that this isoform is also thought to interact with the insulin-stimulated Ras-signaling pathway (31).

To conclude, in rat preadipocytes, the present report i) shows the presence of α, β, ε and ζ PKC isoforms, ii) provides evidence for the possible involvement of ζ PKC in the post-receptor signaling pathway of insulin, the major adipogenic hormone and iii) reveals variable ζ PKC content depending on preadipocyte anatomical origin which suggests that the site-related variations of proliferating and differentiating capacities of preadipocytes may depend, at least in part, on ζ PKC.

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