Association of protein kinase Cα with adducin in 3T3-L1 adipocytes

Palle G. Laustsen a, William S. Lane b, Vann Bennett c, Gustav E. Lienhard a,⁎

a Department of Biochemistry, Vail Building, Dartmouth Medical School, Hanover, NH 03755, USA
b Harvard Microchemistry and Proteomics Analysis Facility, Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA 02138, USA
c Howard Hughes Medical Institute and Department of Cell Biology, Duke Medical Center, Durham, NC 27710, USA

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Abstract

There is evidence that the atypical protein kinases C (PKCα, PKCζ) participate in signaling from the insulin receptor to cause the translocation of glucose transporters from an intracellular location to the plasma membrane in adipocytes. In order to search for downstream effectors of these PKCs, we identified the proteins that were immunoprecipitated by an antibody against PKCα/ζ from lysates of 3T3-L1 adipocytes through peptide sequencing by mass spectrometry. The data show that PKCα is the major atypical PKC in these cells. Moreover, an oligomeric complex consisting of α- and γ-adducin, which are cytoskeletal proteins, coimmunoprecipitated with PKCα. Association of the adducins with PKCα was further indicated by the finding that the adducins coimmunoprecipitated proportionally with PKCα in repeated rounds of immunoprecipitation. Such an association is consistent with literature reports that the adducins contain a single major site for PKC phosphorylation in their carboxy termini. Using antibody against the phospho form of this site for immunoblotting, we found that insulin caused little or no increase in the phosphorylation of this site on the adducins in a whole cell lysate or on the small portion of the adducins that coimmunoprecipitated with PKCα. PKCα and the adducins were located in both the cytosol and subcellular membranous fractions. The binding of PKCα to adducin may function to localize PKCα in 3T3-L1 adipocytes. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Protein kinase C; Adducin; 3T3-L1 adipocyte

1. Introduction

Insulin stimulates glucose transport in fat and muscle cells by stimulating the trafficking of the glucose transporter GLUT4 from its intracellular location to the plasma membrane [1,2]. This trafficking consists of the movement of intracellular storage vesicles containing GLUT4 to the plasma membrane, the docking of these vesicles at the membrane, and the subsequent fusion of the vesicles with the membrane [1,2]. It is not known which step or steps in the trafficking are stimulated by insulin. Moreover, the complete signaling pathway or pathways from the activated insulin receptor to the trafficking process are not known [2,3]. One partial insulin signaling pathway that is known to be required for GLUT4 translocation is the pathway in which insulin activates phosphoinositide (PI) 3-kinase [2,3]. The activation of PI 3-kinase results in the elevation of PI 3,4,5-trisphosphate, which in turn leads to the activation of protein kinase B and of the atypical protein kinases C (PKCα and PKCζ) [4–7]. Some studies
have presented evidence that activation of protein kinase B participates in signaling GLUT4 translocation [8-12], and others have presented evidence that activation of PKCζ participates [13-17]. It may be that both kinases do so. However, the substrate or substrates of these kinases that are on the signal transduction pathway for GLUT4 translocation have not been identified.

Many kinases complex tightly with one or more of their substrates [18]. In the present study we attempted to find substrates of PKCζ in 3T3-L1 adipocytes by the approach of coimmunoprecipitation, with the expectation that one of these substrates might participate in signaling GLUT4 translocation. 3T3-L1 adipocytes were chosen, since these cells show robust insulin-stimulated GLUT4 translocation, and insulin stimulates PKCζ activity in these cells by approx. 2.5-fold [13,15]. Moreover, experiments in both 3T3-L1 adipocytes and rat adipocytes with constitutively active PKCζ, dominant negative PKCζ, and a PKCζ interacting protein have provided evidence that PKCζ participates in signaling GLUT4 translocation [13-17]. Our results show that 3T3-L1 adipocytes express primarily PKCζ and indicate that a substantial portion of the PKCζ is complexed with the cytoskeletal protein adducin.

2. Materials and methods

2.1. Antibodies

An antibody against the carboxy terminal 20 amino acid residues of mouse PKCζ was purchased from Santa Cruz Biotechnology (cat. No. sc216); this antibody also reacts with mouse PKCλ, which differs by two amino acids in this sequence. Rabbit antiserum against the carboxy terminal 204 amino acids of rat γ-adducin [19] was a generous gift from Dr. Susan Jaken at Lilly Research Laboratories. A mouse monoclonal antibody against a phosphopeptide encompassing the PKC phosphorylation site of the adducins was raised against the sequence FRTPphosphoFLKK, which is present in both α- and γ-adducin. It reacts only with α-, β-, and γ-adducin molecules that are phosphorylated on this site (V. Bennett, unpublished results). The details of the preparation and characterization of this antibody will be reported elsewhere.

2.2. Cell culture and immunoprecipitations

3T3-L1 fibroblasts were cultured and differentiated into adipocytes as described previously [20]. Just before use cells were incubated in serum-free medium for 2 h. They were then left in the untreated state or treated with insulin for the time period and at the concentration given in the figure legends. The cells were then washed once with phosphate-buffered saline and lysed at 20°C with 1 ml per 10 cm plate of a lysis buffer consisting of 3% octaethylene glycol decyl ether (C12E9 or Thesit, from Roche Molecular Biochemicals) in 20 mM HEPES, 120 mM KCl, 5 mM MgCl₂, pH 7.4 with phosphatase (10 mM β-glycerol phosphate, 100 mM okadaic acid) and protease inhibitors (1 mM phenylmethanesulfonyl chloride, 10 µM EP475, 10 µM leupeptin, 1 µM pepstatin). The lysate was centrifuged at 20 000×g for 10 min at 4°C to pellet the nuclei. The infranatant was removed from the triglyceride and the pellet and passed through a 0.22 μm filter (Gelman, cat. No. 4192) to remove traces of fat droplets. The lysate cleared in this way was incubated for 2 h with antibody (10 µg anti-PKCζ, 10 µg control rabbit immunoglobulin, or 5 µl antiserum against γ-adducin per ml lysate), and then the immunoadsorbates were collected on protein A-Sepharose (Amersham Pharmacia Biotech) for 1.5 h. The beads were washed four times with lysis buffer, and the adsorbed proteins were released with SDS sample buffer containing 20 mM dithiothreitol and 8 M urea.

2.3. SDS-PAGE and protein sequencing

For analysis by protein staining, the immunoprecipitates were separated by SDS-PAGE on a 5–15% gradient gel, and the proteins were then transferred electrophoretically to nitrocellulose and stained with colloidal gold (Bio-Rad). For amino acid sequencing, PKCζ was immunoprecipitated from 6 ml of lysate, and the proteins in the immunoprecipitate were separated by SDS-PAGE on a 5–15% gradient gel. The gel was stained with colloidal Coomassie blue (Novex), and the protein bands to be sequenced were cut from the gel.
The protein bands were subjected to in-gel reduction, carboxymidomethylation, and tryptic digestion (Promega). Multiple peptide sequences were determined in a single run by microcapillary reverse-phase chromatography directly coupled to a Finnigan LCQ quadrupole ion trap mass spectrometer equipped with a custom nanoelectrospray source. The column was packed in-house with 5 cm of C18 support into a New Objective one-piece 75 μm internal diameter column terminating in a 8.5 μm tip. Flow rate was 190 nl per min. The ion trap was programmed to acquire successive sets of three scan modes consisting of: full scan mass spectrum (MS) over alternating ranges of 395–800 m/z or 800–1300 m/z, followed by two data dependent scans on the most abundant ion in those full scale scans. These data dependent scans allowed the automatic acquisition of a high resolution (zoom) scan to determine charge state and exact mass, and MS/MS spectra for peptide sequence information. MS/MS spectra were acquired with a relative collision energy of 30% and an isolation width of 2.5 Da, and recurring ions dynamically excluded. Interpretation of the resulting MS/MS spectra of the peptides was facilitated by programs developed at the Harvard Microchemistry Facility and by database correlation with the algorithm Sequest [21,22].

2.4. Immunoblotting

Immunoblotting was carried out with samples of the lysates, depleted lysates, and immunoprecipitates described above. In addition, for the experiment described in Fig. 3, 10 cm plates of 3T3-L1 adipocytes were washed with phosphate-buffered saline and solubilized directly in 3 ml of SDS sample buffer with 20 mM dithiothreitol. Proteins were separated by SDS-PAGE and transferred electrophoretically to Immobilon P (Millipore). The membranes were blocked with 5% non-fat dry milk in 150 mM NaCl, 20 mM Tris–Cl, pH 7.4 (TBS) and treated with antibody in 1% milk in TBS at room temperature for 2 h. The concentrations of the antibodies were 1 μg per ml for the antibody against PKCζ, 0.2 μg per ml for the antibody against phosphoadducin, and 1/1000 dilution for serum against γ-adducin. Detection was with horseradish peroxidase-conjugated secondary antibodies or protein A (Bio-Rad) and the enhanced chemiluminescence reagent (Pierce).

2.5. Subcellular fractionation

3T3-L1 adipocytes were fractionated into cytosol, mitochondria/nuclei, plasma membrane, and high and low density microsomes, as described in [23].

3. Results

3.1. Proteins coimmunoprecipitating with PKCζ

In an effort to identify proteins associated with PKCζ in 3T3-L1 adipocytes, we immunoprecipitated PKCζ from lysates of basal and insulin-treated 3T3-L1 adipocytes with an antibody against the carboxy terminal peptide of both PKCζ and PKCζ (see Section 2) and then analyzed the immunoprecipitates for coprecipitating proteins by SDS-PAGE. As a control, immunoprecipitation was performed with irrelevant immunoglobulin. Fig. 1 presents the results of such an experiment. Four protein bands, at approx. 72, 90, 116, and 160 kDa, were

<table>
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<th>Band</th>
<th>Protein</th>
<th>Database GI No.</th>
<th>Size (aa)</th>
<th>Peptides sequenced</th>
<th>Sequence coverage (%)</th>
</tr>
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<td>586</td>
<td>14</td>
<td>37</td>
</tr>
<tr>
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<td>13</td>
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<tr>
<td>3</td>
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<td>1083589</td>
<td>735</td>
<td>12</td>
<td>22</td>
</tr>
<tr>
<td>4</td>
<td>p137</td>
<td>2498734</td>
<td>656</td>
<td>13</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>p160</td>
<td>3327162</td>
<td>1234</td>
<td>11b</td>
<td>15</td>
</tr>
</tbody>
</table>

*aSee Fig. 1.

bThe protein in the table is human p160. Four of the peptides were present in it, and the remaining seven were present in translations of mouse expressed sequence tags. The aa sequences of these expressed sequence tags showed that they were portions of the mouse homologue of human p160.
specific to the PKCα/ζ immunoprecipitate (Fig. 1). Insulin treatment did not result in a significant change in the pattern of coimmunoprecipitating proteins (compare lanes 1 and 2).

The PKCα/ζ immunoprecipitation was performed on a large scale. The four protein bands were isolated from a Coomassie blue-stained gel, and their identities determined by peptide sequencing by mass spectrometry, as described in Section 2. Table 1 summarizes the results. The protein at 72 kDa was PKCα. Although the antibody used for immunoprecipitation reacts with both PKCα and PKCζ, of the 14 peptides sequenced from the 72 kDa band, 13 were unique to PKCα, one was common to PKCα and PKCζ, and none was unique to PKCζ. Since PKCα and PKCζ migrate at the same position upon SDS-PAGE [15], this finding indicates that PKCα is much more abundant than PKCζ in 3T3-L1 adipocytes. Kotani et al. have reached a similar conclusion from analysis of 3T3-L1 adipocytes for the two kinases by Northern and immunoblotting [15].

The protein band at 90 kDa was identified as γ-adducin, and the band at 116 kDa consisted of a mixture of α-adducin and another protein, previously designated p137. There are three members of the adducin family, designated α, β, and γ; they are closely related in amino acid sequences [24]. The adducins exist as heterodimers and heterotetramers consisting of either the α/β or α/γ forms [24]. Thus, the finding of both α- and γ-adducin in the PKCα immunoprecipitate is consistent with this information on the oligomeric structures of the adducins. The Coomassie blue staining intensities of the PKCα, γ-adducin, and α-adducin bands in the preparative gel used for sequencing were about the same (data not shown). Similarly, the colloidal gold staining intensities of these three bands in the analytical gel shown in Fig. 1 (lane 1) were about the same. These observations suggest that a substantial portion of the immunoprecipitated PKCα was complexed with α/γ-adducin. However, the presence of p137 in the α-adducin band and of a weakly stained background band arising from the antibody against PKCα in the γ-adducin band (see lanes 1 and 5 of Fig. 1) precludes precise determination of the relative amounts of the three proteins in the PKCα immunoprecipitate.

The p137 protein is the mouse homologue of human p137, a protein of unknown function that was cloned from a human colon cDNA library [25]. This protein has been shown to be an extracellular protein that is linked to the plasma membrane by a glycosylphosphatidylinositol linkage in human epithelial cells [25]. Because of this location, it seems unlikely that p137 would be associated with PKCα in intact 3T3-L1 adipocytes. For this reason we have not investigated p137 further.

The protein band at 160 kDa, which we have designated p160, was identified as the mouse homologue of a human protein of unknown function whose cDNA has been cloned as part of a large-scale cDNA sequencing project [26]. This protein has a predicted binding domain for the compound FK506 near its amino terminus [27]. We raised an antiserum against the peptide LQGNSRRLSLTPDPEK from
mouse p160 and affinity purified the antibody. The affinity-purified antibody both immunoblotted and immunoprecipitated p160 from a 3T3-L1 adipocyte lysate. However, when an immunoprecipitate of p160 from a lysate of the adipocytes was immunoblotted for PKC\(\alpha\), no PKC\(\alpha\) was detected (data not shown). In a further effort to determine whether PKC\(\alpha\) and p160 were associated, we performed three rounds of immunoprecipitation of PKC\(\alpha\) on a lysate of 3T3-L1 adipocytes and examined both the immunoprecipitates and depleted lysates from each round for both PKC\(\alpha\) and p160 by immunoblotting. As expected, the amount of PKC\(\alpha\) in both the immunoprecipitate and the depleted lysate from each round was less than that from the previous round. However, only a very small fraction of the p160, approx. 4%, was coimmunoprecipitated in each round, and the amount of p160 in the PKC\(\alpha\) immunoprecipitate and the depleted lysate did not detectably decrease after each round of immunoprecipitation (data not shown). These results, especially the finding of a constant amount of coimmunoprecipitated p160 with each round despite decreasing amounts of immunoprecipitated PKC\(\alpha\), suggest that the antibody against PKC\(\alpha\) bound directly to p160. Thus, cross-reaction of p160 with the antibody against PKC\(\alpha\), rather than association of p160 with PKC\(\alpha\), is probably the explanation for the coimmunoprecipitation of p160. In this regard, by computer analysis we could not find a sequence in p160 with detectable similarity to the peptide used to generate the antibody against PKC\(\alpha\).

Because of the relatively large amounts of the antibody heavy and light chains in the SDS samples in Fig. 1, any protein of 60 kDa or less that coimmunoprecipitated with PKC\(\alpha\) might have been obscured by the strong staining of the antibody chains. In order to examine the low molecular weight region for coimmunoprecipitating proteins, the experiment described in Fig. 1 was performed, except that the immunoprecipitate was solubilized at 20°C in SDS sample buffer with 8 M urea and without reductant. The unreduced antibody ran as a broad, heavily stained band in the region between 90 and 200 kDa, and consequently it did not interfere with detection of lower molecular weight proteins. The only protein of low molecular weight detected in the PKC\(\alpha\) immunoprecipitate was one of approx. 55 kDa, and it was present in one experiment but not in a repetition. This protein was sequenced and found to be the lipoamide acyl transferase component precursor of the branched chain \(\alpha\)-ketoacid dehydrogenase complex (GI No. 1709438, size 482 amino acids (aa)). Since this enzyme is mitochondrial and since PKC\(\alpha\) is not present in the mitochondria (see Fig. 5), the two proteins are unlikely to be associated in the intact cell.

### 3.2 Association of the adducin with PKC\(\alpha\)

In order to investigate the potential interaction of adducin with PKC\(\alpha\) more fully, we immunoprecipitated PKC\(\alpha\) and adducin from lysates of basal and insulin-treated 3T3-L1 adipocytes, and then immunoblotted the lysates, depleted lysates, and immuno-

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![Fig. 2. Coimmunoprecipitation of adducin with PKC\(\alpha\). (A) Lysates of 3T3-L1 adipocytes in the basal state or after treatment with 166 nM insulin for 10 min were immunoprecipitated with antibody against PKC\(\alpha\) or \(\gamma\)-adducin as described in Section 2. Aliquots of the lysates (Lys), depleted lysates (dLys), and immunoprecipitates (IP) were immunoblotted for PKC\(\alpha\) and \(\gamma\)-adducin. The 1× load is an amount derived from 1.5% of a 10 cm plate of adipocytes. (B) A lysate of adipocytes in the basal state was subjected to three rounds of immunoprecipitation with antibody against PKC\(\alpha\) or irrelevant rabbit immunoglobulin (IgG) as the control. The initial lysate and the depleted lysates and immunoprecipitates from each round were immunoblotted for PKC\(\alpha\) and \(\gamma\)-adducin. The 1× load was derived from 1.5% of a 10 cm plate. Repetitions of these experiments gave similar results.](image-url)
precipitates for PKC\(\alpha\) and adducin (Fig. 2A). The antibody against PKC\(\alpha\) immunoprecipitated approx. 50% of the PKC\(\alpha\) (Fig. 2A, compare lanes 1, 2 to lanes 3, 4). As expected, the PKC\(\alpha\) immunoprecipitate contained adducin (Fig. 2A, lanes 5, 6). However, the amount of adducin that coimmunoprecipitated with PKC\(\alpha\) was only approx. 10% of the total (compare lanes 5, 6 to lanes 1, 2). Although in Fig. 2A it appears that insulin treatment led to a slight increase in the amount of adducin in the PKC\(\alpha\) immunoprecipitate (compare lanes 5 and 6), in two other experiments there was no difference in the amount of coimmunoprecipitating adducin in samples from basal and insulin-treated cells (for example, see Fig. 4B). The antibody against adducin precipitated approx. 50% of the adducin (compare lanes 7, 8 with 1, 2). However, no PKC\(\alpha\) was detected in the adducin immunoprecipitate (lanes 9, 10).

One possible explanation for the absence of PKC\(\alpha\) in the adducin immunoprecipitate is that the binding sites on adducin for PKC\(\alpha\) and the antibody against adducin overlap, such that both cannot bind simultaneously. In fact, the antibody against \(\gamma\)-adducin used here is against its carboxy terminal 204 amino acids, and as described below, the PKC phosphorylation site in \(\gamma\)-adducin is located near its carboxy terminus. An alternative explanation is that the antibody against PKC\(\alpha\) cross-reacts directly with adducin, even though by computer analysis neither \(\alpha\)-nor \(\gamma\)-adducin contains a peptide sequence with any similarity to that of the PKC\(\alpha\) peptide used to generate the antibody against PKC\(\alpha\). In order to distinguish between these possibilities, we performed three rounds of immunoprecipitation of PKC\(\alpha\) and analyzed the depleted lysates and immunoprecipitates from each round for PKC\(\alpha\) and adducin by immunoblotting (Fig. 2B). As expected, the amount of PKC\(\alpha\) in the depleted lysate and in the immunoprecipitate decreased with each round of immunoprecipitation (Fig. 2B, lanes 1–6 and lanes 7–9). Moreover, the amount of adducin that coimmunoprecipitated with the PKC\(\alpha\) also decreased with each round in proportion with PKC\(\alpha\) (lanes 7–9). On the other hand, the amount of adducin in the depleted lysate decreased only slightly, since only a small portion of the adducin was immunoprecipitated (lanes 1–6 and lanes 7–9). These results indicate that adducin was immunoprecipitated because it was associated with PKC\(\alpha\), rather than bound directly to the antibody against PKC\(\alpha\). If the latter were the case, it would be expected that approximately the same amount of adducin would have been immunoprecipitated in each round, since depleted lysate continued to contain most of the adducin. The conclusion that adducin associates directly with PKC\(\alpha\) is consistent with an earlier study in which another PKC isoform, PKC\(\alpha\), was shown to bind to a fragment consisting of the carboxy terminal 204 aa of \(\gamma\)-adducin in a blot overlay assay [28]. However, coimmunoprecipitation of adducin with any PKC isoform has not been found previously.

### 3.3. Phosphorylation of adducin

The three adducins are known substrates for PKC. The site of PKC phosphorylation is a serine residue in the carboxy terminus, which is nearly identical in the three adducins [24]. The carboxy terminus is highly positively charged; in \(\alpha\)-adducin its sequence is KKKKFRTPS*FLKKSKKKSDS. The serine undergoing phosphorylation, which is marked by *, is referred to as the RTPS site. As noted in Section 1, insulin is reported to activate PKC\(\alpha\) in 3T3-L1 adipocytes. Maximal activation occurs at 5 min after insulin treatment and is approx. 2.5-fold [15]. Consequently, since adducin associates with PKC\(\alpha\), it seemed likely that it might show increased phosphorylation on the RTPS site in response to insulin. In order to test this possibility, SDS lysates were prepared from basal and insulin-treated 3T3-L1 adipocytes and immunoblotted with an antibody specific for the phosphorylated RTPS site. Fig. 3 presents the
results from four separate experiments in which the cells were treated with insulin for 5 min. α-Adducin was more highly phosphorylated than γ-adducin. In experiments 1 and 2 insulin caused no increase in phosphorylation, whereas in experiments 3 and 4 insulin treatment appeared to cause a slight increase in phosphorylation. In another experiment the phosphorylation of adducin after insulin treatment for 2, 5, 10, 30, and 60 min was examined, and the insulin effect at the other times was no greater than that at 5 min (data not shown). From these data we conclude that insulin caused little or no increase in adducin phosphorylation on the RTPS site. As a control for the effectiveness of the insulin treatment, it was shown by immunoblotting for phosphotyrosine that the insulin treatment elicited the phosphorylation of the insulin receptor and insulin receptor substrate 1 (data not shown). Although insulin caused no significant increase in phosphorylation of the adducin on the RTPS site, adducin was susceptible to further phosphorylation on this site. Treatment of the cells with phorbol myristate acetate at 1 μM for 15 min caused an approx. 3-fold increase in phosphorylation of the RTPS site on both adducins (data not shown). Phorbol myristate acetate activates the conventional and novel PKCs [29], and it may also activate PKCβ, since phorbol myristate acetate elevates PI 3,4,5-trisphosphate in 3T3-L1 adipocytes [53].

It seemed possible that the small portion of the adducin that was associated with PKCβ might exhibit insulin-stimulated phosphorylation. Consequently, we immunoprecipitated PKCβ from basal and insulin-treated adipocytes and then immunoblotted the associated adducin for phosphorylation on the RTPS site (Fig. 4). In fact, insulin caused no detectable increase in phosphorylation of the associated adducin. The ratio of phosphorylated γ-adducin to α-adducin was higher in the coimmunoprecipitating adducin than in the total pool of adducin (compare Figs. 3 and 4). Equal amounts of γ-adducin were present in the PKCβ immunoprecipitates from basal and insulin-treated cells (Fig. 4, lower blot).

3.4. Subcellular distributions of PKCβ and adducin

In order to determine the extent to which PKCβ and adducin are colocalized, we examined the subcellular distributions of the two proteins (Fig. 5). PKCβ was approximately equally concentrated in the cytosol, plasma membranes, and low density microsomes. By contrast adducin was most concentrated in the low density microsomes, and was also abundant in the cytosol, plasma membranes, and high density microsomes.

The low density microsomes consist of a mixture of vesicles, largely derived from the endosomes, and cytoskeletal elements; the high density microsomes consist largely of endoplasmic reticulum and Golgi [30,31]. Since the lanes in Fig. 5 were loaded with equal amounts of protein, the relative intensities depict the relative concentrations in the various frac-
tions. The relative amounts of protein recovered in the cytosol, plasma membrane, and high and low density microsomes were approx. 1.0, 0.03, 0.10, and 0.12, respectively (data not shown). Consequently, because the amount of the cytosol protein is 8-fold or more than that in the other fractions, most of both the PKCζ and adducin was in the cytosol. Insulin treatment caused no significant change in the subcellular distribution of either protein. The subcellular distribution of PKCζ and the lack on an insulin effect upon it are in agreement with the results of previous studies, although in these the PKC was probably mistakenly identified as the closely related ζ isoform [13,32].

4. Discussion

The search for interacting proteins by means of coimmunoprecipitation has the advantage that it provides a display of the associated proteins and their relative amounts in the cell type of interest. Our present results indicate that the major protein interacting with PKCζ in 3T3-L1 adipocytes is α/γ-adducin. A large number of proteins have been previously found to be associated with PKCζ/ζ in a variety of cell types, but this is the first report of an interaction with adducin. The previously identified interacting proteins include: Ras [33], protein kinase B [34], p70 S6 kinase [35], protein kinase PDK1 [5–7], casein kinase 2 [36], IκB kinase [37], 14-3-3 β/δ [38], fibroblast growth factor receptor substrate 2 [39], a protein induced during apoptosis known as par-4 [40], α-interacting protein [41], a human homologue of the Caenorhabditis elegans polarity determinant par-6 [42], a human homologue of the C. elegans unc-76 protein involved in axonal outgrowth [43], the VHL tumor suppressor protein [44], a rat homologue of the C. elegans polarity protein par-3 [17], and a protein variously named ZIP, p62, EBIAP, and A170 [45]. It is somewhat surprising that none of these 16 other proteins was found to coimmunoprecipitate with PKCζ from the lysates of 3T3-L1 adipocytes. There are several possible explanations. The interacting protein may not be expressed in 3T3-L1 adipocytes, or the protein may be expressed, but only a small portion of PKCζ associates with it. Also, some of these proteins may interact with PKCζ through the carboxy terminus of the latter. Since the antibody used here for immunoprecipitation of PKCζ is against this region, it would not be able to precipitate the PKCζ with such a protein associated. For example, it has been shown that the kinase PDK1 associates with PKCζ by association with its carboxy terminus [7]. In this regard, to our knowledge it has not been previously noted that the carboxy terminal sequences of PKCζ and PKCζ, which are EESV and EECV, respectively, are similar to the consensus sequence of a PDZ binding motif, which is ESXV [46]. The mammalian homologues of the C. elegans par-3 and par-6 proteins that interact with PKCζ/ζ have PDZ domains [17,42].

The adducin heterodimer is a cytoskeletal protein that binds to the ends and sides of actin filaments, and recruits the protein spectrin to these sites [24,47]. Phosphorylation of the adducins by PKC at the carboxy terminal RTPS site inhibits the actin binding and spectrin recruiting activities [47]. Moreover, phosphorylation at this site correlates with changes in the subcellular distribution of adducin in vivo [47,48]. Our finding that PKCζ was associated with adducin initially suggested that insulin treatment might result in substantially enhanced phosphorylation of adducin via activation of PKCζ. However, insulin treatment of the 3T3-L1 adipocytes resulted in only a small increase in phosphorylation of the total adducin at the RTPS site, and no increase was detected for the small portion of adducin associated with PKCζ. The small increase in phosphorylation of the total adducin could thus be due to its phosphorylation either by PKCζ and/or by another PKC isoform that was activated by insulin. There is evidence that insulin slightly activates PKCζ and PKCζ in 3T3-L1 adipocytes [13].

It remains possible that the activation of PKCζ by insulin has effects on adducin phosphorylation and function that were not detected by the approaches used here. The study showing adducin phosphorylation by PKC at the RTPS site employed a preparation of purified rat brain PKC that probably consisted of a mixture of the conventional PKC isotypes [24]. It is possible that PKCζ, which is an atypical PKC, phosphorylates a different site on the adducins. The preferred substrate sequence motifs for conventional and atypical PKCs have been found...
to be similar, but not identical [49]. If this were the case, increased phosphorylation of adducin would not have been detected with the phosphospecific antibody for the RTPS site. A second possibility is that an increase in the rate of phosphorylation at the RTPS site in response to insulin is matched by an increase in the rate of dephosphorylation, such that there is an increase in flux through the site but no net increase in phosphorylation. Finally, it may be that the small increase in phosphorylation of total adducin at the RTPS site in response to insulin reflects a much larger increase in phosphorylation at this site in a small portion of the adducin at a specific critical location in the cell.

Insulin enhances actin assembly at the periphery of 3T3-L1 adipocytes and causes membrane ruffling [50]. There is evidence that this effect, like the GLUT4 translocation, is downstream from PI 3-kinase [51]. Moreover, an intact actin cytoskeleton is required for insulin-stimulated GLUT4 translocation in 3T3-L1 and rat adipocytes, since depolymerization of actin filaments with cytochalasin D or latrunculin inhibits this process [51,52]. The interaction of PKCα with adducin may function to localize PKCα to the cytoskeleton, and this interaction may participate in some way in the signaling of membrane ruffling and GLUT4 translocation.

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