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DIRECT EVIDENCE FOR PROTEIN KINASE C INVOLVEMENT IN INSULIN-STIMULATED HEXOSE UPTAKE

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Insulin has been reported to translocate protein kinase C (PKC) in rat adipocytes, and activation of PKC by phorbol esters is known to increase hexose uptake in these cells (1,2). To test the hypothesis that PKC may participate in insulin-stimulated hexose uptake, adipocytes were partially depleted of protein kinase C by overnight phorbol ester treatment, thereby impairing insulin effects on hexose uptake. Purified PKC was then introduced into these PKC-depleted adipocytes by electropermeabilization, and this fully restored insulin-stimulated hexose uptake. These findings provide direct evidence that PKC is required for insulin-stimulated hexose uptake.

Insulin activates, and/or stimulates the translocation of, PKC in a variety of cell types, including rat adipocytes (1,3-9). PKC activation is associated with increases in glucose tranport, and insulin effects on this process are inhibited by PKC inhibitors (10,11), and, in certain cell types (2,12-14), by phorbol ester-induced PKC depletion. Nevertheless, the role of PKC in insulin-stimulated glucose transport has remained uncertain, since these experimental approaches are indirect. Presently, we attempted to directly examine the involvement of PKC in insulin-stimulated glucose transport by electroporating purified PKC into PKC-depleted rat adipocytes.

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Materials and Methods

Adipocyte preparation: Rat adipocytes were prepared by collagenase digestion of rat epididymal fat pads obtained from 200-250 g male Holtzman rats fed *ad libitum* as described (1). Adipocytes were cultured overnight at 37°C in 93% air/7% CO_2 in Dulbecco's minimum essential medium (DMEM) (Gibco) as a 10% (vol/vol) suspension containing 5 mM glucose, 0.5% BSA, 10 mM HEPES, and 20.5 units/ml penicillin and 20.5 μg/ml streptomycin (see15). Where indicated, 500 nM 12-0-tetradecanoylphorbol-13-acetate (Sigma) was present during overnight incubations.

PKC preparation: For PKC repletion studies, in preparation A, PKC was purified first by chromatography of rat brain cytosol on a Mono Q (Pharmacia) column. [An FPLC system (Pharmacia) (see16) was used for all column procedures.] PKC-containing fractions from six Mono Q runs were pooled and chromatographed on a spherical-type hydroxyapatite (HAP) column (Regis). HAP column fractions containing PKC were pooled, concentrated by ultrafiltration and dialyzed overnight at 4°C in DMEM prior to use in electropermeabilization studies. More highly-purified PKC (preparation B) was prepared using first DEAE-cellulose (Whatman DE52), followed by Mono Q, phenylsuperose (Pharmacia), and HAP column chromatography. Pure PKC was then stored in a 50% glycerol solution at -70° C and dialyzed as described above. After this 4-step column purification, a single band of silver staining was seen at 80-85 kDa on SDS-PAGE, indicating that the PKC was purified to homogeneity or near-homogeneity.

Electropermeabilization: Electropermeabilization (EP) was accomplished by using 0.8 ml of a 50% suspension of adipocytes in DMEM containing 0.1% BSA and, where indicated, purified rat brain PKC (1000 mU). Cells were placed in cuvettes and subjected to one discharge of 0.35 kV/cm at 960μF capacitance, for 10-12 msec in a Bio-Rad Gene Pulsar equipped with a capacitance extender.

Hexose Uptake: To study hexose uptake, adipocytes were washed three times and suspended (6%, vol/vol) in glucose-free Krebs Ringer phosphate buffer (KRP) containing 1% BSA, and 260 μl aliquots were placed in plastic tubes. After equilibration for 30 min, adipocytes were treated without or with 3 nM insulin (Elanco) for 30 min, and 1 min uptake of [1,2-^{3}H]2-deoxyglucose ([^{3}H]2DOG; ICN; 0.1 μCi/per tube; 0.05 mM) was determined (17). Cytochalasin B (70 μM) was used to determine specific glucose transporter-mediated uptake, and as an indicator of cell integrity. Cytochalasin B blanks ranged from 100-150 cpm (irrespective of treatments) and were subtracted from each group.

Immunoreactive PKCβ: Immunoreactive PKCβ was measured on cytosolic extracts (5 μg protein) as described (24). PKCβ antiserum (21) was a gift from Drs. Bryan Roth and John Mehegan.

Results and Discussion

Rat adipocytes exhibit losses of PKC and insulin- and phorbol ester-stimulated glucose transport following several hours (14), or overnight (15), treatment with phorbol esters. With overnight phorbol ester treatment, we have

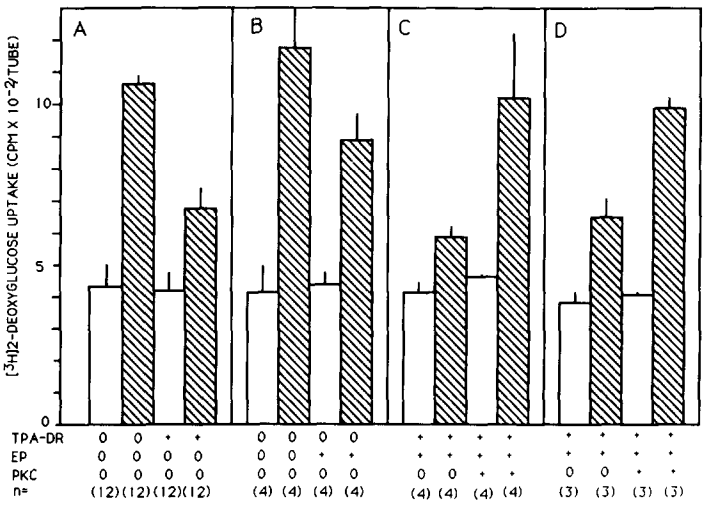

Figure 1: Restoration of insulin effects in phorbol ester-downregulated rat adipocytes by protein kinase C. Adipocytes were prepared and cultured as described. Where indicated (TPA-DR), 500 nM TPA was present during overnight incubations. PKC from preparation A was used in experiments shown in panel C, and PKC from preparations B was used in experiments depicted in panel D. Electropermeabilization (EP) was performed in the presence or absence of purified PKC (1000 U). Control (open bars) and insulin-stimulated [^{3}H]2-DOG uptake (hatched bars) are expressed as mean ± SE of (n) separate experiments, each performed in quadruplicate.

reported (15) that PKC enzymatic activity and PKC-β immunoreactivity are decreased by 50-70%, and this is associated with 55-65% losses in insulin-stimulated [^{3}H]2-DOG uptake. Presently, using identical conditions, after overnight culture, insulin stimulated the uptake of [^{3}H]2-DOG by 2-3 fold in control, non-downregulated adipocytes (Fig. 1A) (comparable stimulatory effects of insulin in cultured adipocytes have been noted by other workers - see (18)). As in previous studies (14,15), when adipocytes were cultured in the presence of 500 nM 12-0-tetradecanoylphorbol-13-acetate (TPA), insulin-stimulated [^{3}H]2-DOG uptake was decreased by 50%, although basal uptake was not altered (Fig. 1A). Simple electropermeabilization of control, non-downregulated, cultured adipocytes mildly inhibited insulin-stimulated, but not basal, [^{3}H]2-DOG uptake (Fig. 1B). In TPA-downregulated, cultured adipocytes, simple electropermeabilization decreased the insulin response further to 15% of that observed in non-downregulated cells, without affecting basal 2-DOG uptake (Fig 1C). In contrast, when TPA-downregulated rat adipocytes were electropermeabilized in the presence of purified rat brain PKC, insulin-stimulated 2-DOG uptake was restored nearly fully to the original response observed in non-downregulated, non-electropermeabilized, cultured adipocytes (Fig. 1C and D for electropermeabilization in the presence of PKC preparations A and B, respectively). It should be noted that basal hexose uptake was not

Table 1: Effects of insulin on [^{3}H]glycerol incorporation into diacylglycerol in rat adipocytes cultured with or without TPA

Treatment	[^{3}H]glycerol incorporation into diacylglycerol	
	Control Adipocytes	Adipocytes Downregulated with TPA
	(cpm/tube)	
None	680±33 (7)	550±1 (3)
3nM insulin	869±58 (8)	866±67 (4)

Adipocytes were cultured overnight with or without 500 nM TPA as described in Fig. 1, then washed, resuspended (6% vol/vol) in glucose-free KRP containing 1% BSA, and 0.5 ml of the cell suspension was incubated for 30 min with or without 3nM insulin and 10 μCi of [2-^{3}H]glycerol (S.A. 30 Ci/mmol; ICN). Incorporation of label into diacylglycerol was determined as described (19). Values are mean ± SE of (n) determinations.

timulated by electropermeabilization in the presence of PKC. A heat-inactivated reparation of purified PKC had no effect on basal or insulin-stimulated [^{3}H]2-OG uptake. Thus, the PKC preparations did not contain a factor that, of itself, ould stimulate hexose uptake in the absence of insulin.

The fact that the PKC-dependent increase in 2-DOG uptake was observed nly in insulin-treated cells suggested that this increase in 2-DOG uptake is lependent on insulin-induced activation of a process that requires PKC for its xpression. An obvious candidate for this process is diacylglycerol (DAG) eneration, and, indeed, we found that insulin stimulated [^{3}H]glycerol-labeling of AG, regardless of whether or not the cultured rat adipocytes were TPA-lownregulated (Table 1) (19). The intactness of the insulin effect on [^{3}H]DAG-roduction also attests to the fact that TPA-downregulated adipocytes can espond very well to insulin, at least in steps that are proximal to PKC activation. Ve have also demonstrated intactness of insulin receptors and insulin-timulated tyrosine kinase activity in TPA-downregulated, cultured rat dipocytes (15, 20).

Two methods were used to purify the rat brain PKC that was used in lectroporation experiments. Preparation A (see Fig. 1C) utilized a 2-step hromatographic method, and resulted in partial purification of PKC. Preparation 3 (see Figs. 1D and 2), utilized a 4-step chromatographic method, and PKC was urified to homogeneity or near homogeneity. [Note: both PKC preparations ontained PKC isoforms α, β, δ, ε and ζ, all of which are present in rat adipocytes 20).] Results from experiments using preparations A and B were identical in hat insulin-stimulated [^{3}H]2-DOG uptake in TPA-downregulated cells could be estored to the full, non-downregulated level following electropermeabilization f adipocytes in the presence of either PKC preparation. We confirmed that the ighly purified PKC (preparation B) was indeed introduced into adipocytes by

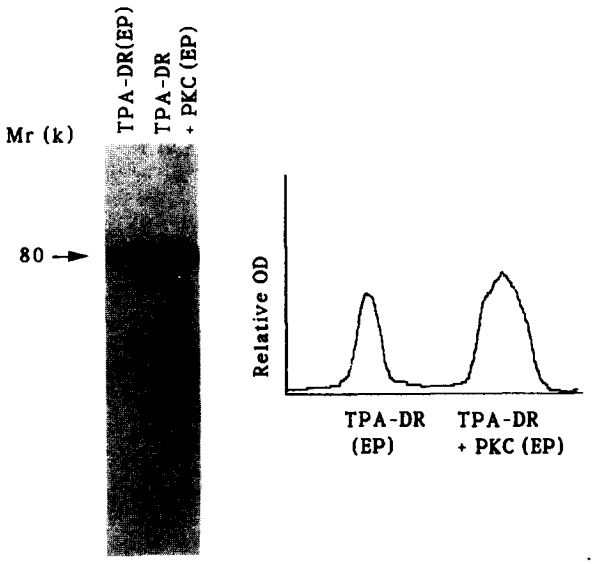

Figure 2: Incorporation of protein kinase C into rat adipocytes by electropermeabilization. Adipocytes were prepared as described in Fig. 1. TPA-DR (EP) indicates electropermeabilized adipocytes downregulated by culturing overnight in the presence of 500 nM TPA, and TPA-DR + PKC (EP) indicates TPA-DR adipocytes electropermeabilized in the presence of PKC. Immunoreactive PKCβ was determined by immunoblotting (left). Densitometric scans (right) of immunoreactive PKCβ are shown relative to TPA-DR (EP) adipocytes. Similar increases in immunoreactive PKC (greater than 50%) were noted in three immunoblots.

the electropermeabilization method (Fig. 2). In this particular experiment, insulin-stimulated glucose transport decreased by 83% following TPA-induced downregulation and subsequent electropermeabilization. Electropermeabilization in the presence of PKC restored over 75% of the insulin response, without affecting basal [^{3}H]2-DOG uptake. The actual introduction of PKC into these adipocytes was evaluated immunologically, using antisera specific for PKCβ (21), and a 53% increase in PKCβ immunoreactivity was noted (Fig. 2). This increase in cellular PKC was slightly less than the percent increase in glucose transport, but this non-proportionality may reflect that other contributing PKC isoforms are present in the rat brain preparations and are not recognized by the PKCβ antiserum, or that some adipocyte PKC-β pools are not active in support of glucose transport.

In contrast to adipocytes, TPA-induced PKC downregulation does not inhibit insulin-stimulated glucose transport in certain cell types (22,23). This failure to desensitize insulin effects has been studied extensively in BC3H-1 myocytes, and there is complete loss of PKCα following chronic TPA treatment, but no appreciable loss of PKCβ, which remains responsive to insulin, but is poorly responsive to acute TPA stimulation (24). The ability of rat adipocytes

and possibly other rodent tissues, e.g., the soleus muscle (12,13), to desensitize their hexose uptake responses to insulin more effectively in response to chronic TPA treatment may be due to sufficient downregulation of specific PKC isozymes (in particular, PKCβ) that seem to be more relevant in mediating the effects of insulin-stimulated diacylglycerol on glucose transport. Further studies are needed to evaluate the role of individual PKC isozymes in insulin-stimulated glucose transport.

The present results provide the first direct evidence that PKC is required for insulin-stimulated glucose transport in rat adipocytes. However, the reason for the PKC requirement remains to be determined. Haring and co-workers (25,26), have proposed a two-step process that insulin may use to regulate glucose transport in rat adipocytes, viz., a) a largely PKC-dependent, or PKC-mimicked, mechanism that involves translocation of glucose transporters from an intracellular site to the plasma membrane, and b) a PKC-independent mechanism that results in activation of translocated glucose transporters. [Note: As will be reported elsewhere, we have observed that insulin translocates the insulin regulatable glucose transporter, Glut4, from microsomes to plasma membranes in cultured rat adipocytes nearly as well as in freshly isolated adipocytes.] If this model is correct, the present finding, viz., that replenishment of cellular PKC can totally, rather than partially, restore insulin-stimulated glucose uptake in downregulated adipocytes, suggests that the PKC-dependent step, presumably glucose transporter translocation, is the initial step, and is essential for expression of the second activation step. Further studies will be needed to directly test this possibility.

In summary, purified PKC restores insulin effects on glucose transport in TPA-downregulated rat adipocytes. This provides the first direct evidence that PKC is an essential requirement or mediator for insulin-stimulated glucose transport.

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