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Protein kinase C(19–31) pseudosubstrate inhibition of insulin action in rat adipocytes

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Treatment of intact adipocytes with the autoregulatory PKC pseudosubstrate PKC(19–31) inhibited insulin-stimulated hexose uptake and lipogenesis, with no effect on basal values. The effect was dose-dependent with respective IC_{50} values of 30 μM and 600 μM for insulin-stimulated hexose uptake in electroporated and intact adipocytes. These studies indicate that PKC may play a role in the mediation of insulin action in adipocytes.

Insulin; Protein kinase C; Hexose uptake; Adipocyte

1. INTRODUCTION

Protein kinase(s) C constitutes a family of Ca^{2+}, phospholipid-dependent serine/threonine specific kinases which regulate multiple cellular processes and are activated by diacylglycerol, acting as a 'second messenger' for cell surface interactive stimuli [1]. Recent studies have shown that insulin stimulates PKC translocation from the cytosol to the particulate fraction in adipocytes [2,3], suggesting that PKC is involved in mediating insulin action. Several approaches have been used to investigate the role of PKC in insulin-stimulated signal transduction, including prolonged incubation with phorbol esters to 'down-regulate' PKC [4], as well as the use of biochemical and biological inhibitors of the regulatory and catalytic domains of PKC [5,6]. Each of these experimental designs, while useful, is somewhat limited. It is now becoming increasingly recognized that chronic incubation with biologically active phorbol esters may lead only to selective isozyme down-regulation, and that even partial retention of PKC may be sufficient to fully evoke the agonist effect in some cell types [7]. Furthermore, the use of inhibitors of PKC may be difficult to interpret due to possible non-specific inhibition of other cellular kinases.

Kemp and House [8] have reported that cytosolic PKC activity is autoregulated (inhibited) by an 18 amino acid sequence (PKC(19–36)), found in the highly conserved amino-terminus, which functions as a pseudosubstrate for the carboxy-terminus catalytic site. Since it has been shown that this pseudosubstrate motif, as well as a 13 amino acid subset (PKC(19–31)), is a highly specific competitive inhibitor of PKC [8], this suggested that the pseudosubstrate could be used to investigate the role of PKC in insulin or other hormone action. In this study, we have studied the effects of PKC(19–31) on insulin-stimulated lipogenesis and glucose uptake in intact and electroporated rat adipocytes.

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Abbreviations: PKC, protein kinase C; BSA, bovine serum albumin; DOG, deoxyglucose; PMA, phorbol myristate acetate; INS, insulin; KRP, Krebs-Ringer phosphate

2. MATERIALS AND METHODS

2.1. *Materials*

[^{3}H]2-DOG (30 Ci/mmol) was purchased from New England Nuclear (Boston, MA) and [6-^{3}H]D-glucose (38 Ci/mmol) and Cyto-Scint scintillation cocktail were from ICN Biomedicals (Costa Mesa, CA). Insulin was purchased from ELANCO (Indianapolis, IN) and PMA was from Sigma. Collagenase was from Worthington (Freehold, NJ). PKC(19-36) was purchased from Bachem (Torrance, CA). Synthetic peptide amides, corresponding to the PKC sequence 19–31 as well as an unrelated 15 amino acid sequence (ALDTNYSFSSTEKNC) corresponding to the N-terminal 15 amino acids of transforming growth factor-β, were synthesized using t-Boc chemistry and PAM resins by established methods [9,10] employing an automated peptide synthesizer (model 430A; Applied Biosystems Inc., Foster City, CA). At the end of the synthesis the peptides were cleaved from the resin by anhydrous liquid hydrogen fluoride and desalted by gel filtration.

2.2. *Methods*

Isolated rat adipocytes were prepared from nonfasted male Holtzman rats (150–200 g body weight) by collagenase treatment [11]. Cells (6% suspension in KRP + 1% BSA) were pre-equilibrated at 37°C to restore adenosine levels, treated in the presence or absence of the peptide sequences for 30 min and then in the presence or absence of 3 nM insulin or 500 nM PMA for an additional 30 min. One min

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[^{3}H]2-DOG uptake was determined by centrifugation over silicone oil as previously described [6,12]. (Addition of 100 μM phenylisopropyl adenosine did not alter control or stimulated uptake values.) In some experiments, 50% suspensions of adipocytes were electroporated in KRP-buffer containing 5 mM glucose and 1% BSA, in the presence or absence of PKC(19-31), using a Bio-Rad Gene Pulser with a Bio-Rad Capacitance Extender. Adipocytes were electroporated for 10-15 ms at 350 mV and 960 μF of capacitance. Isolated adipocytes were subsequently washed and diluted to a 6% suspension in glucose-free KRP buffer containing 1% BSA and the appropriate peptide concentrations maintained. Insulin (3 nM) was added for 30 min and one min [^{3}H]2-DOG uptake determined. Following electroporation, there was a small compromise in the effect of insulin on [^{3}H]2-DOG uptake. However, insulin effects were still readily demonstrable, e.g., in 8 experiments values for the mean ± SE were 295 ± 40 cpm vs 1117 ± 157 cpm (control vs insulin in non-electroporated cells) and 334 ± 44 cpm and 833 ± 88 cpm (control vs insulin in electroporated cells).

Lipogenesis was determined by incorporation of [6-^{3}H]glucose into the lipid fraction of the adipocytes. Briefly, 0.5 ml of a 6% adipocyte suspensions were incubated in the presence or absence of PKC(19-31) for 30 min after which 3 nM INS was added to selected samples for 10 min, and then 1 μCi of [6-^{3}H]D-glucose (0.1 mM glucose, final concentration) was added to all samples which were incubated for an additional 30 min. The reaction was terminated by the addition of 2 ml cold methanol, and lipids were extracted with 4 ml chloroform. The chloroform/methanol (2:1) mixture was washed 3 times with 2 ml water and 400 μl aliquots of the chloroform extracts were dried and the lipid-incorporated radioactivity determined.

3. RESULTS

3.1. *Effects of PKC (19-31) pseudosubstrate on insulin and PMA stimulated 2-DOG uptake in intact adipocytes*

As shown in Fig. 1 (left panel), PKC(19-31) pseudosubstrate caused a dose-dependent inhibition of both insulin- and PMA-stimulated hexose uptake in isolated intact adipocytes. In this experiment, insulin and PMA stimulated 2-DOG uptake by 5-fold and 2-fold, respectively. PKC(19-31), which is a subset of the pseudosubstrate amino acid sequence found in the highly conserved regulatory domain of PKC isozyme, inhibited both insulin- and PMA-stimulated hexose uptake. Incubation with 80 μM PKC(19-31) had little or no effect on PMA- or insulin-stimulated hexose uptake, while full inhibition was noted following a 30 min incubation with 800 μM PKC(19-36) pseudosubstrate. Incubation with PKC(19-31) did not inhibit basal [^{3}H]2-DOG uptake.

3.2. *Effect of PKC(19-31) on insulin-stimulated lipogenesis in intact adipocytes*

As further shown in Fig. 1 (right panel), 3 nM insulin provoked a 4-fold increase in the incorporation of [6-^{3}H]D-glucose into the lipid fraction of isolated adipocytes. PKC(19-31) inhibited insulin-stimulated lipogenesis in a pattern analogous to insulin-stimulated hexose transport. PKC(19-31) had little effect on basal levels of lipogenesis, had little effect on insulin action at 80 μM PKC(19-31), but was fully effective in blocking insulin-stimulated lipogenesis at 800 μM pseudosubstrate.

3.3. *Effects of various peptides on insulin-stimulated hexose uptake in intact adipocytes*

As shown in Fig. 2, in studies utilizing intact adipocytes, PMA-stimulated hexose uptake was more sensitive to PKC(19-31) inhibition than was insulin-stimulated hexose uptake: respective IC_{50}s for PMA and insulin were 300 μM and 600 μM PKC(19-31). The entire pseudosubstrate amino acid sequence described

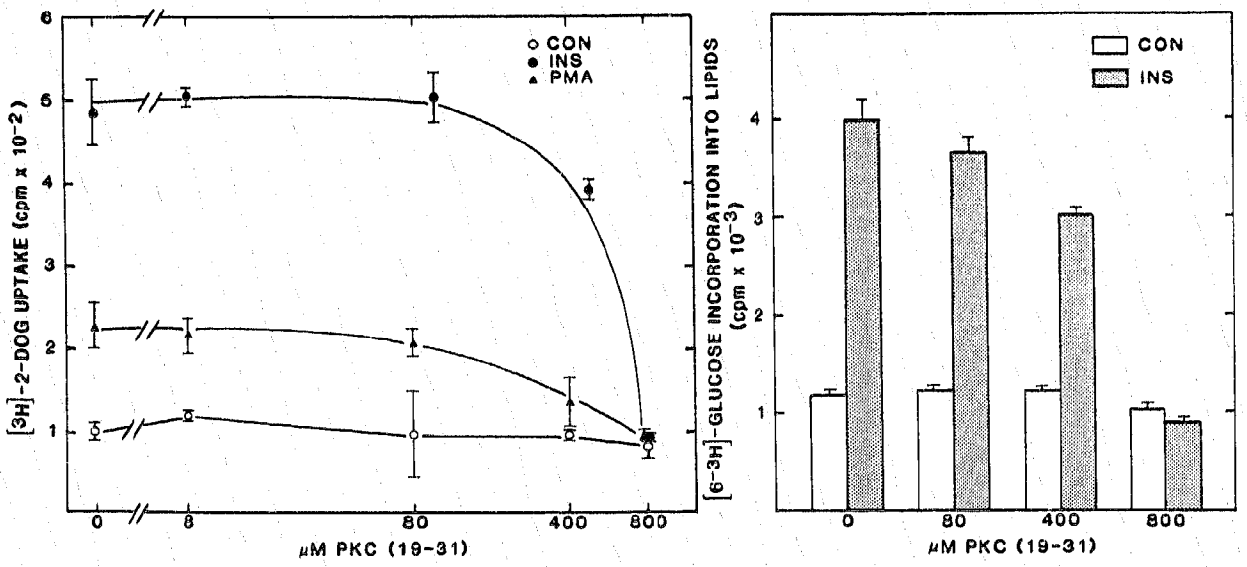

Fig. 1. Dose-dependent effects of PKC(19-31) on insulin- and PMA-stimulated 2-DOG uptake (left panel). Dose-dependent effects of PKC(19-31) on insulin-stimulated lipogenesis in intact adipocytes (right panel). Values are the mean ± SE of triplicate determinations from representative experiments.

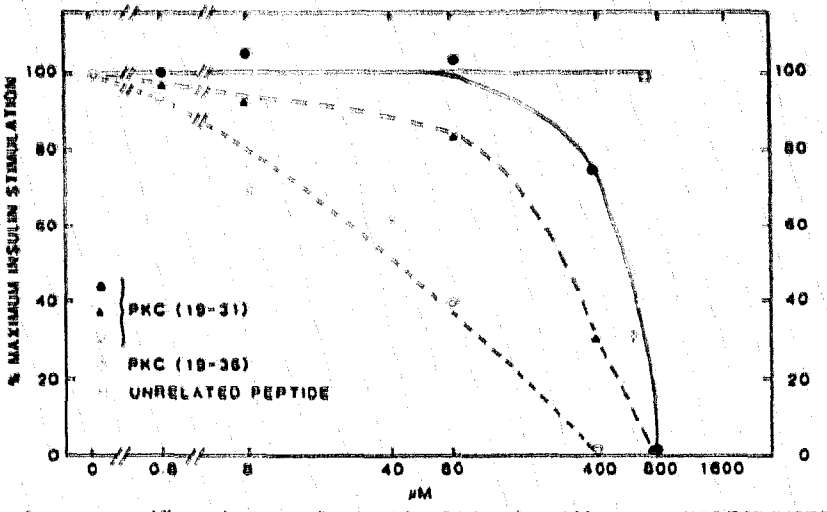

Fig. 2. Effect of PKC pseudosubstrates, specific analogues and an unrelated 15 amino acid sequence (ALDTNYSFSSTEKNC) corresponding to the N-terminal 15 amino acids of transforming growth factor-β on 500 PMA- (▲) or 3 nM insulin (○,●,○,◇)-stimulated hexose uptake in electroporated (----) or intact adipocytes. Values are based on 3-4 determinations in a representative experiment from a series of 3-4 similar experiments. (Values for PKC(19-36) are based on duplicate determinations from a single experiment, due to limited material.)

by House and Kemp, PKC(19-36), also blocked insulin-stimulated 2-DOG uptake to approximately the same extent as PKC(19-31). Each largely blocked the insulin effect at 600-800 μM concentrations. Incubation with an unrelated amino acid sequence corresponding to the N-terminal 15 amino acids of transforming growth factor-β had no effect on insulin-stimulated hexose uptake.

3.4. *Effect of PKC(19-31) on insulin-stimulated hexose transport in electroporated rat adipocytes*

When adipocytes were electroporated in the presence of PKC(19-31), insulin-stimulated hexose transport was markedly more sensitive to PKC(19-31), with an IC_{50} of 30 μM PKC(19-31) compared to 600 μM in intact adipocytes (Fig. 2).

4. DISCUSSION

Several studies have now suggested a role for PKC in insulin-stimulated hexose transport in isolated rat adipocytes. Insulin-stimulates PKC activity [13,14] and translocation [2,3] in these cells. Furthermore, treatment with activators of PKC including exogenous diacylglycerols [15,16], phospholipase C, which increases endogenous diacylglycerol [16], and biologically active phorbol ester diacylglycerol analogues [17] each stimulate hexose transport in the adipocyte in an insulin-like manner. Recently, Haring and colleagues [18] have proposed a two-step model for insulin-action in adipocytes in which PKC is involved in insulin-stimulated recruitment of glucose transporters from the microsomal fraction to the plasma membrane.

In this investigation, we have studied the effect of a specific PKC inhibitor on insulin-stimulated hexose transport. House and Kemp [8] have reported that a specific, arginine-rich sequence, PKC(19-36), within the highly conserved C1 region of PKC, inhibits the serine/threonine kinase activity of the catalytic domain. In our study, we have shown the incubation of either intact or electroporated adipocytes with PKC(19-31), also an effective pseudosubstrate sequence [8], blocked insulin- and PMA-stimulated hexose transport. PMA-stimulated hexose uptake was somewhat more sensitive to PKC(19-31) than was the insulin effect on hexose uptake, with respective IC_{50}s of 300 μM and 600 μM. This difference in insulin and PMA sensitivity is consistent with that previously observed for other PKC inhibitors, viz. H-7, sangivamycin, and staurosporine in adipocytes, and is consistent with a two-step model in which insulin activation of PKC may be involved primarily in mediating insulin-stimulated glucose transporter translocation with insulin further activating glucose transporters by a PKC-independent mechanism. Alternatively, the differential sensitivities to inhibitors may reflect agonist specific, i.e. PMA vs diacylglycerol (insulin), effects on specific PKC isozyme activation. Along the latter lines, PKC inhibitors are equally effective when insulin concentrations are used which evoke glucose transport responses comparable to that of PMA [6]. Additionally, as noted with other PKC inhibitors [6], the inhibitory effect of PKC(19-31) was quite sharp with the full effect of the inhibitor evidenced over a single order of magnitude.

Relatively high molecular concentrations of pseudosubstrate were required to inhibit PMA or insulin action in intact adipocytes. However, when electroporated adipocytes were treated with PKC(19-31) the effective concentration required to inhibit insulin-stimulated hexose uptake was reduced 10-20-fold, from

an IC_{50} of 600 μM in intact adipocytes to an IC_{50} of 30 μM in electroporated adipocytes. The latter value is in reasonable agreement with results others have observed in cell-free systems. For example, a concentration of 3 mM PKC(19-31) delivered by microelectrode impalement with a 30-min diffusion time was required to consistently inhibit long-term potentiation of synaptic transmission [19], while 20 min treatment with 100 μM PKC(19-31) failed to block long-term potentiation following tetanic stimulation. Additionally, the IC_{50} for PKC(19-31)inhibition of γ-chain phosphorylation in streptolysin-*O*-permeabilized T-lymphocytes is 7-20 μM [20], and retroinverso PKC(19-31) inhibited purified brain PKC phosphorylation of glycogen synthase 1-12 at an IC_{50} of 31 μM [21]. These differences in observed IC_{50}s most probably reflect the variable affinity of PKC isozymes for the phosphorylation of specific substrates. Whereas the IC_{50} for PKC(19-31) using a synthetic sequence of glycogen synthase and purified rat brain PKC is 90 nM PKC pseudosubstrate [8], the IC_{50} for inhibition by PKC(19-31) is 1 μM using purified PKC from EL4 thymoma cells and histone IIIS substrate [22]. Additionally, as evidenced by the increased efficacy of PKC(19-31) in electroporated cells, the higher pseudosubstrate concentrations required for inhibition of insulin action in intact adipocytes may reflect permeability factors, as well as the presence of multiple PKC cellular substrates, with varying affinities for PKC-mediated phosphorylation. Indeed, uptake of radiolabeled inositol was increased from 0.2% of the applied radioactivity in intact cells to 2.5% in electroporated cells [23]. This indicates that electroporation increases cellular uptake of the applied pseudosubstrate; however, intracellular concentrations of PKC(19-31), while increased, may not attain extracellular levels due to the transient nature of membrane pore formation. In our study, the cytochalasin B values for 2-DOG uptake in intact and electroporated adipocyte were almost identical indicating rapid resealing of the electroporated-induced membrane pores (143 ± 11 cpm per assay in intact adipocytes and 169 ± 5 cpm per assay in electroporated adipocytes).

As recently demonstrated by Krebs and colleagues PKC(19-31) is a more selective inhibitor of PKC than the more widely used inhibitors staurosporine and H-7 and has no effect in vitro on either ribosomal S6 kinase or myelin basic protein kinase at concentrations below 1 mM [22]. In conclusion, our findings indicate that PKC(19-31) can effectively be used to study the role of PKC in hormone action in intact and electroporated rat adipocytes and provide additional evidence indicating that PKC may be involved in insulin-stimulated hexose transport and lipogenesis in isolated rat adipocytes.

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