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Insulin, oxytocin, and vasopressin stimulate protein kinase C activity in adipocyte plasma membranes

(phorbol esters/Western blot analysis/subcellular fractionation)

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ABSTRACT **Incubation of isolated rat adipocytes with insulin, vasopressin, or oxytocin increased plasma membrane-bound protein kinase C (PKC) activity by 100–400%. PKC activity was assayed by a procedure that is virtually background-free, thus permitting assay of protein kinase activity in highly diluted samples of solubilized membranes. Hormone-dependent increases in PKC activity were limited to plasma membranes. Stimulation of the kinase was half-maximal with 70 pM insulin, and the hormone effect was rapid. Oxytocin and vasopressin produced effects on PKC similar to insulin, but the magnitude of the vasopressin stimulation exhibited seasonal variations. Treatment of cells with phorbol 12-myristate 13-acetate (PMA) resulted in a loss of PKC activity from the cytosol and a gain in plasma membrane activity, indicative of translocation of the enzyme. With activity measurements it was not possible to determine if insulin stimulated a translocation of the kinase. However, Western blot analysis of plasma membranes with polyclonal antibodies directed against PKC suggest that at least some of the insulin-stimulated PKC activity resulted from enzyme translocation.**

In 1966 Rodbell (1) noted that "there is some common action of oxytocin, insulin and phospholipase C," based on the observation that, when applied extracellularly, the three agents increased glucose transport and amino acid incorporation into proteins of fat cells. A possible basis for this mimicry of hormones by exogenous phospholipase C lies in the finding that many hormone receptors increase phospholipid turnover, production of diacylglycerol, and activation of the calcium- and phospholipid-dependent enzyme, protein kinase C (PKC) (2–4), and there is indirect evidence for PKC participation in acute metabolic effects of insulin on adipocytes and other cells. Tumor-promoting phorbol esters, which substitute for diacylglycerol in stimulating PKC (3), increase transport of glucose (5–9) and amino acids (7), activate pyruvate dehydrogenase (7), increase lipogenesis (6, 8, 9–11), and stimulate ion transport (12, 13). Moreover, insulin has been reported to increase phospholipid metabolism (14–16) and the formation of diacylglycerol in adipose tissue (17) and in cultured myocytes (17–19), but there is sharp disagreement on this issue (20, 21).

Despite the evidence implicating PKC in the acute actions of insulin on adipocytes, data demonstrating increased PKC activity in adipocyte membranes after insulin treatment are notably lacking. In contrast to reported findings of insulin-stimulated increased PKC activity in cultured smooth muscle cells (22) and rat diaphragm (23), several laboratories have reported that insulin does not increase PKC activity in plasma membranes of isolated fat cells or cultured 3T3-L1 adipocytes after acute exposure to the hormone (12, 24–26). On the other hand, decreased phorbol ester binding to adipocyte plasma membrane sites, thought to represent PKC, has been observed after treatment of fat cells with insulin (27).

Like phorbol esters, vasopressin and oxytocin mimic a number of insulin effects on adipocytes (for examples, see refs. 1, 28–30). However, in contrast to insulin, stimulation of phospholipid metabolism and diacylglycerol formation in adipocytes by vasopressin and oxytocin is readily detected (20, 21). Moreover, PKC has been implicated in the actions of these nonapeptide hormones in a variety of cells (for examples, see refs. 31–34). Nevertheless, other than a report on activation of hepatic PKC with vasopressin (35), there appear to be no reports that directly demonstrate increased PKC activity in isolated membranes after exposure of adipocytes or other types of cells to either vasopressin or oxytocin.

In this paper, with the use of a highly sensitive protein kinase assay method (36), we report that exposure of isolated rat adipocytes to insulin, vasopressin, or oxytocin increases PKC activity in adipocyte plasma membranes.

METHODS

Cell Preparation, Incubations, and Subcellular Fractionation. Adipocytes were isolated from the epididymal fat pads of 180- to 200-g Sprague–Dawley rats according to Rodbell (28) as modified (37). Adenosine (200 nM) was included in all incubation media (38). Adipocytes were incubated at 2×10^6 cells per ml [10% (vol/vol)] for 30 min in the presence of the hormones or phorbol 12-myristate 13-acetate (PMA) at concentrations indicated in the legends to the figures and tables.

After incubation, the cells were washed once with homogenization buffer [20 mM Tris·HCl, pH 7.4/255 mM sucrose/2 mM EDTA/2 mM EGTA/2 mM dithiothreitol/leupeptin (10 μg/ml)/aprotinin (1 trypsin inhibitor unit/ml)/0.1 mM phenylmethylsulfonyl fluoride] that had been equilibrated at 18°C. Each milliliter of fat cells was homogenized in 5 ml of medium, and subcellular fractions were prepared (39). For PKC assays, membranes were solubilized at a protein concentration of 0.5–1 mg/ml for 60 min at 4°C in the homogenization medium containing 1% Triton X-100; this step was performed immediately upon completion of the fractionation procedure. Enzyme samples were diluted by at least 1:20 into the homogenization buffer without detergent and assayed immediately. The further 1:10 dilution in the assay reaction

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Abbreviations: PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PS, phosphatidylserine; DO, 1,2-diolein.

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mixture yielded final protein concentrations of <5 μg/ml; typically, 150–250 ng of protein was used per determination.

Assay of PKC. The PKC assay medium contained 10 mM Tris·HCl (pH 7.5), 5 mM magnesium acetate, 0.3 mM $CaCl_2$ (0.1 mM Ca^{2+} in excess of EGTA carried over from the enzyme medium), 20 μM ATP, 0.5 μCi of [γ-^{32}P]ATP (1 Ci = 37 GBq), and 200 μM GS peptide as the substrate (36). (GS peptide is a synthetic dodecapeptide of glycogen synthase, NH_2-Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ala-Ala-Lys-Lys-COOH.) Preliminary studies showed that this substrate concentration was optimal for expressing PKC activity. Phosphatidylserine (PS) and 1,2-diolein (DO) (Avanti Polar Lipids) were prepared as described (36) and added at final concentrations of 100 and 10 μg/ml, respectively. "Basal" is defined as the activity in the presence of EGTA but without the combination of Ca^{2+} plus the lipid mixture. The final assay volume was 100 μl, of which 10 μl was the adipocyte subcellular fractions as described above. After incubation for 20 min at 30°C, the reaction was stopped and the ^{32}P-labeled GS peptide was purified as described (36). Assay background values were <20 cpm per 10^6 cpm of [γ-^{32}P]ATP in the reaction mixture.

RESULTS

Insulin Stimulation of PKC Activity in Adipocyte Plasma Membranes. Exposure of adipocytes to increasing insulin concentrations led to increased PKC activity in solubilized plasma membranes (Fig. 1). Compared to membranes from control cells, membranes from cells incubated with maximal insulin concentrations exhibited a 150% increase in the component of activity dependent on the presence of Ca^{2+} plus the lipid mixture containing PS and DO [(Ca^{2+} + PS/DO)-dependent activity]. The half-maximal insulin concentration for increased PKC activity was approximately 70 pM (10 microunits/ml). There was no change in kinase activity by insulin in assays conducted with EGTA alone.

The insulin-dependent increase in PKC activity was rapid (Fig. 2). Adipocytes were incubated with a supramaximal insulin concentration and, at the indicated times, the cells were introduced into the homogenization medium at 18°C. An insulin-induced increase in plasma membrane PKC activity was evident at the earliest time tested, 0.5 min. However, from the times the cells were removed from the incubation medium, introduced into the homogenization medium, and washed, 1.5–2 min elapsed. Thus, if one includes this washing period, the earliest time tested was 2–2.5 min. By 4 min after initiation of incubations with insulin, or 6 min including the washing time, activation of PKC was maximal. Similar to the data in Fig. 1, insulin increased PKC activity in solubilized plasma membranes by >100%.

Inclusion of EDTA and EGTA in the homogenization and subcellular fractionation media was essential for retaining the insulin-mediated stimulation. Omission of both chelators led to a 40–50% loss of PKC activity and all of the insulin effect. Similar losses occurred if membranes were frozen prior to solubilization (data not shown).

Comparison of Effects of Insulin, Vasopressin, Oxytocin, and PMA on PKC Activity. Incubation of isolated fat cells with either vasopressin or oxytocin increased the (Ca^{2+} + PS/DO)-dependent activity in plasma membranes by approximately 200% (Fig. 3), which was less than the insulin effect for the experiments presented in Fig. 3 (see below). PMA

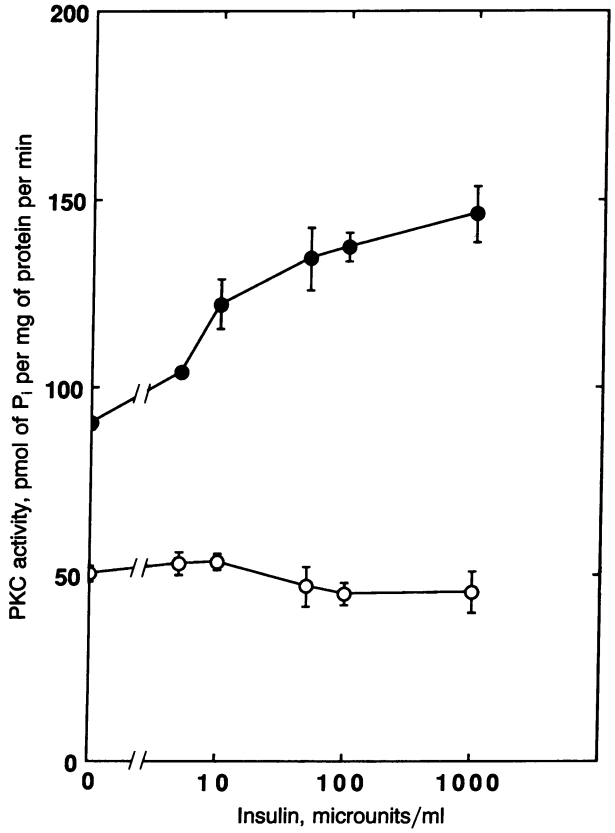

FIG. 1. Concentration dependence of insulin-stimulated increases in adipocyte plasma membrane PKC activity. Isolated adipocytes were incubated for 30 min at 37°C with insulin as indicated. Cells were homogenized and plasma membranes were prepared. PKC activity was measured on solubilized plasma membrane samples in the presence of 2 mM EGTA (open circles) or of Ca^{2+} + PS/DO (solid circles). Data are mean ± SEM (n = 6) of triplicate kinase determinations from two experiments.

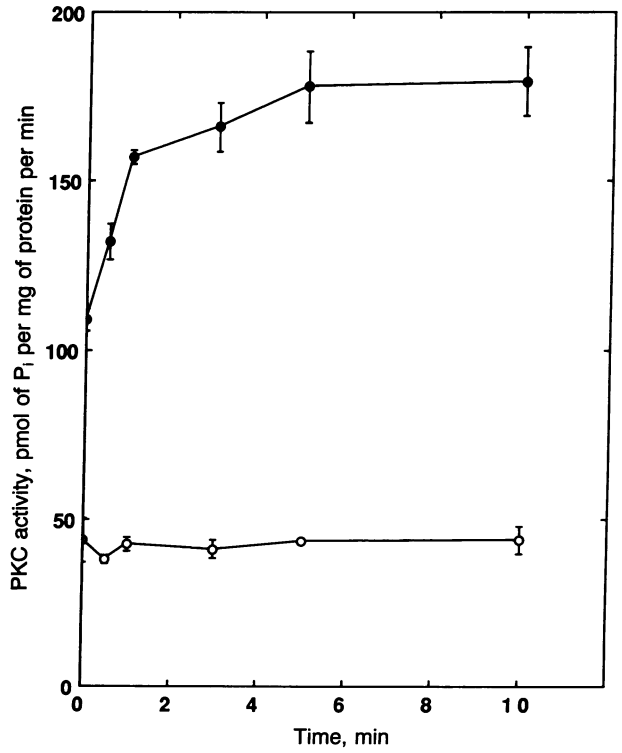

FIG. 2. Time course of insulin-dependent stimulation of PKC in adipocyte plasma membranes. Isolated adipocytes were incubated with 700 nM insulin at 37°C as indicated. Cells were homogenized, plasma membranes were prepared, and PKC activity in solubilized membranes was determined. Open circles represent activity in the presence of 2 mM EGTA, and solid circles represent activity in the presence of Ca^{2+} + PS/DO. Data are mean ± SEM (n = 6) of triplicate kinase determinations from two experiments.

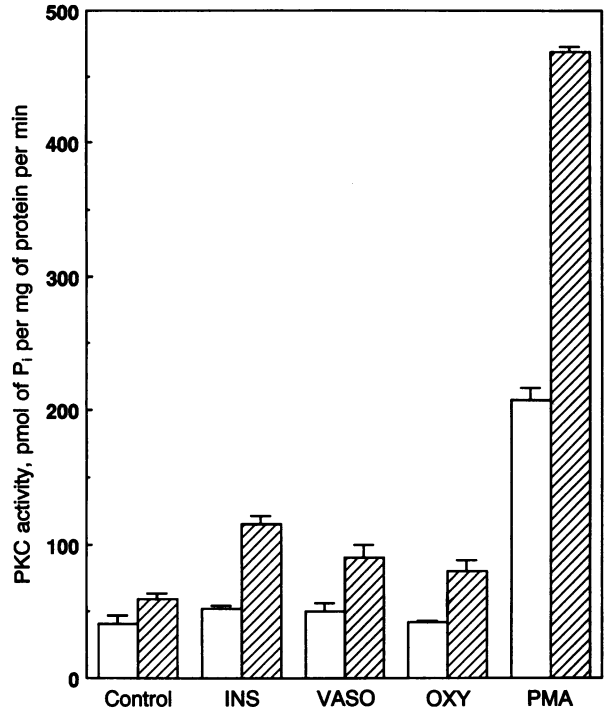

FIG. 3. Effect of various hormones and agents on stimulation of PKC activity in adipocyte plasma membranes. Isolated adipocytes were incubated for 30 min at 37°C with the indicated hormones and agents at the following concentrations: 700 pM insulin (INS), 10 μM vasopressin (VASO), 10 μM oxytocin (OXY), and 1 μM PMA. Cells were homogenized, plasma membranes were prepared, and PKC activity was measured in the presence of 2 mM EGTA (open bars) or of Ca^{2+} + PS/DO (hatched bars). Data are mean ± SEM (n = 6) of triplicate kinase determinations from two experiments.

increased basal activity by a factor of 4 and (Ca^{2+} + PS/DO)-dependent activity by a factor of 14, values considerably greater than those obtained with the hormones.

Although the magnitude of the insulin response remained relatively constant over a 1-year period, the vasopressin response varied from nil during late autumn and winter to twice the insulin response during the summer (data not shown).

Subcellular Distribution of PKC Activity in Control and Hormone-Treated Adipocytes. The effects of vasopressin, insulin, and PMA on the subcellular distribution of GS-peptide phosphorylating activities were examined (Table 1). In contrast to other experiments in this study, the protein kinase assays shown in Table 1 were performed with and without the PS/DO mixture, but Ca^{2+} was present throughout. By the criterion of stimulation by PS/DO, increases in activity in cells treated with PMA, vasopressin, and insulin were limited to the plasma membranes. It is also seen that insulin and vasopressin increased PS/DO-dependent activity by a factor of 5 over control membranes, from approximately 10–50 pmol of P_i transferred per mg of protein per min.

For the experiments shown in Table 1, the cytosolic fractions from control cells exhibited both higher total activity and greater specific activity than all membrane fractions, 5800 pmol of P_i transferred per min and 730 pmol of P_i transferred per mg of protein per min, respectively. Incubation of hormones led to no detectable loss in cytosolic PKC activity, but PMA produced a 60% loss. Only 25–40% of the lost cytosolic activity was recovered in the plasma membranes (data not shown).

Western Blot Analysis of Plasma Membranes. Plasma membranes from control cells and cells incubated with insulin and PMA were probed with polyclonal anti-PKC antibodies (40). Densitometric scanning of autoradiograms of Western blots revealed that insulin increased PKC in the membranes by 34 ± 3% and PMA increased PKC by 97 ± 24% (mean ± SEM; n = 4).

DISCUSSION

The present data demonstrate that insulin, vasopressin, and oxytocin increase plasma membrane-bound protein kinase activity in adipocytes, expression of which is evident upon addition of Ca^{2+} plus the lipid mixture containing PS and DO (Ca^{2+} + PS/DO), well-established PKC cofactors. We cannot state with certainty whether all increases in protein kinase activities elicited by the hormones represent PKC exclusively. On the other hand, we find that the increases in activity measured with PS/DO alone, with (Ca^{2+} + PS/DO), and with Ca^{2+} alone (data not shown) exhibit identical substrate requirements and identical concentration dependencies for inhibition by the PKC inhibitor, H-7 [1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride; data not shown]. Moreover, hormone-induced increases in activity measured under the above conditions are paralleled by phorbol ester-stimulated activity, the only difference being that the phorbol ester effects are greater in magnitude. Thus, it is unlikely that other protein kinases contributed significantly to the activities detected under our assay conditions.

Although the responses to vasopressin and oxytocin were not explored in detail, examination of the insulin concentration dependency and the rate of onset of the reaction indicate that PKC activation is mediated by the insulin receptor. Furthermore, comparison of various well-characterized subcellular fractions (39) show that the hormonal increases in PKC occur at the plasma membrane.

Typically, activation of PKC by various hormones in their respective target cells is revealed after ion-exchange chromatography of solubilized membranes (41). Apparently, insulin-mediated activation of adipocyte PKC is not detected by these standard procedures (12, 24) or by alternate methods (25, 26). It is possible that the positive effects reported in this

Table 1. Subcellular distribution of GS-peptide phosphorylating activities from adipocytes incubated with various agents

	Protein kinase activity, pmol of P_i per mg of protein per min					
	High-density microsomes		Low-density microsomes		Plasma membranes	
Agent added	Ca^{2+}	Ca^{2+} + PS/DO	Ca^{2+}	Ca^{2+} + PS/DO	Ca^{2+}	Ca^{2+} + PS/DO
None	17 ± 2	19 ± 2 (12)	29 ± 5	23 ± 2 (25)	41 ± 2	51 ± 5 (86)
PMA	54 ± 1	80 ± 9 (51)	74 ± 3	67 ± 8 (75)	250 ± 11	560 ± 24 (952)
Vasopressin	25 ± 4	28 ± 1 (18)	50 ± 2	46 ± 3 (52)	81 ± 3	128 ± 4 (218)
Insulin	31 ± 1	35 ± 5 (22)	52 ± 3	52 ± 1 (58)	70 ± 2	126 ± 3 (214)

Cells were incubated (30 min at 37°C) with the indicated agents and subcellular fractions were prepared. Protein kinase activity was measured on Triton X-100-solubilized membranes. Assays were conducted with 0.1 mM free calcium alone (Ca^{2+}) or with PS/DO (Ca^{2+} + PS/DO). Values are mean ± SEM (n = 6) of triplicate determinations from replicate experiments. Values in parenthesis represent total protein kinase activity (pmol of P_i per min) for each membrane fraction assayed with Ca^{2+} + PS/DO. Protein kinase activity in a mixed nuclear/mitochondrial fraction was nondetectable.

study stem from the use of a protein kinase assay method that, because of its extraordinary sensitivity (36), permits measurements of activity in highly diluted samples. Indeed, to detect PKC activity, it was necessary to dilute the enzyme samples considerably, since the adipocyte subcellular fractions contain a substance strongly inhibitory toward both endogenous and purified exogenous PKC (unpublished data).

With activity measurements, we could find no evidence for translocation by insulin, but the activity gained by plasma membranes is but a fraction of the total adipocyte activity, approximately 1%. Although Western blots show that insulin increases the PKC content in plasma membranes, the 34% gain over control membranes seen by Western blot analysis is considerably less than the activity enhancement that, depending on the criteria employed, ranges from at least 100% to as much as 400%. One possible explanation for this discrepancy is that, as with a number of different cells, adipocytes contain multiple forms of PKC (3), only one of which is translocated by the insulin receptor. However, isozyme selectivity would not seem to explain a similar discrepancy in membranes from PMA-treated cells, in which Western blot data showed an apparent doubling in detectable PKC, far less than the typically greater than 10-fold increase in activity. It should be noted that receptor-mediated activation of membrane-bound PKC may occur without apparent translocation (42, 43). Interestingly, the insulin-induced increase in PKC activity in diaphragm and cultured myocytes is not accompanied by a parallel loss in cytosolic activity (22, 23). Until the PKC isozymes in adipocytes are identified, quantitated, and localized, our data should be considered as suggestive of translocation of PKC by insulin.

Although the mechanism(s) whereby the insulin receptor might stimulate PKC remains in question, evidence is mounting for PKC participation in some physiological responses to the hormone. In addition to the mimicry of insulin by phorbol esters in several physiological responses, as discussed above, insulin and phorbol esters increase the phosphorylation of identical substrates in cellular (44) and membrane (45) studies. Interestingly, although Blackshear *et al.* (46) emphasized the differences between phorbol esters and insulin in stimulating the phosphorylation of an 80-kDa protein in 3T3-L1 cells, they also noted that PMA and insulin stimulate the phosphorylation of several proteins in common in 3T3-L1 fibroblasts, adipocytes, and H4IIEC3 hepatoma cells. Also, the ability of sphingosine and several analogs to inhibit insulin-stimulated hexose transport (47) and lipogenesis (48) parallels their inhibition of PKC. On the other hand, PKC activation does not fully mirror the insulin effect, as shown by differences between phorbol ester- and insulin-stimulated translocation of the glucose transporter and activation of glucose transport (49). We find that, although translocation of glucose transporters to the plasma membrane by PMA is one-half the translocation response elicited by insulin, PMA stimulation of glucose transport activity is merely 10% of that produced by insulin. Upon addition of insulin to adipocytes in which a substantial number of transporters have undergone translocation to the plasma membrane by prior incubation with PMA, the rate at which glucose transport activity rises is greater than upon addition of insulin to control cells (J.S., A. D. Habberfield, J.J.E., C.L., I.A.S., and S. W. Cushman, unpublished work). Such data support the proposal that insulin, by PKC activation, might stimulate translocation of transporters but activate the transporters by a separate mechanism (49). In any event, it seems reasonable to conclude that PKC activation is an early event mediated by occupation of the adipocyte insulin receptor and this enzyme should be added to the list of protein kinases that catalyze phosphorylation of serine and threonine residues after insulin receptor activation (50, 51).

In view of the stimulation of phosphatidylinositol breakdown (20) and diacylglycerol formation (21) by vasopressin and oxytocin action on fat cells, the finding that these hormones increase PKC activity is not surprising. Certainly, PKC activation might explain the overlapping adipocyte responses to insulin, vasopressin, oxytocin, and phorbol esters (see above). Interestingly, with respect to glucose transporter translocation versus glucose transport activity, vasopressin and oxytocin effects precisely parallel those of the phorbol ester described above (unpublished data). Finally, the variability in the vasopressin-stimulated PKC response, especially vis-a-vis that of insulin, provides a dramatic example of a seasonal phenomenon, the basis of which remains a mystery, and highlights the value of monitoring hormonal responses over extended periods (38).

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