Redistribution of Phospholipid/Calcium-dependent Protein Kinase and Altered Phosphorylation of Its Soluble and Particulate Substrate Proteins in Phorbol Ester-treated Rat Pancreatic Acini

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ABSTRACT

The biological activity of phorbol esters, such as 12-O-tetradecanoylphorbol-13-acetate, have been associated with activation of phospholipid/Ca\(^{2+}\)-dependent protein kinase. Treatment of rat pancreatic acini with 12-O-tetradecanoylphorbol-13-acetate (10\(^{-8}\) M) resulted in a sustained translocation of phospholipid/Ca\(^{2+}\)-dependent protein kinase activity to the membrane site. The pattern of phosphorylation of at least two substrate proteins (M, 22,000 and 18,000) for this Ca\(^{2+}\)-dependent protein kinase was also altered following exposure to phorbol ester, these phosphoproteins disappearing from the soluble fraction and appearing in the particulate. Concurrently, 12-O-tetradecanoylphorbol-13-acetate stimulated amylase release from intact acini in a time- and dose-dependent fashion. These results suggest a potential role for phospholipid/Ca\(^{2+}\)-activated protein kinase in the regulation of pancreatic exocrine function.

INTRODUCTION

TPA\(^2\) is the most potent derivative of the phorbol diester tumor promoters, possessing the capability of inducing a variety of biological effects in vitro. These effects include changes in phospholipid metabolism and protein synthesis (7, 25, 26), altered rates of DNA synthesis (9), induction of ornithine decarboxylase activity (8), effects upon polyamine biosynthesis (20), changes in cell morphology (17, 23), and phosphorylation of cellular proteins (3, 5, 22). Ultimately, these changes may result in an alteration in gene expression and cellular proliferation, as well as other cellular activities.

Recently, high-affinity binding sites for TPA have been found in various mammalian cell types (4, 15, 24). Using cultured cells, Neidel et al. (18) and others (2, 12) have presented evidence associating the receptor for TPA with the PL/Ca-PK (C-kinase) and further proposed that this kinase is the membrane target of this phorbol ester or shares a close association within the membrane to the receptor. Castagna et al. (2), using purified PL/Ca-PK, demonstrated that TPA directly activates the kinase, in a fashion analogous to diacylglycerol, presumably by binding to the hydrophobic region of the molecule.

Several studies (2, 16, 19) have provided evidence demonstrating that the degree of activation of PL/Ca-PK in vitro and in vivo by phorbol esters correlates with the biological potency of the individual derivatives in tumor promotion. However, even in light of these findings, our understanding of the precise molecular events associated with and subsequent to the binding of TPA remains largely speculative.

An initial study undertaken in our laboratory has shown that TPA induces translocation of assayable PL/Ca-PK activity from the cytosol of pancreatic acinar cells, concomitant with stimulation of amylase secretion (29). Herein we seek to demonstrate a specific TPA-induced association of PL/Ca-PK activity with the membrane compartment of pancreatic acini. Also, we have examined Ca\(^{2+}\)-activated phosphorylation of specific endogenous acinar cell proteins following exposure to phorbol ester. Our results demonstrate that TPA treatment induces rapid and sustained changes in the pattern of appearance of both PL/Ca-PK and phosphorylated endogenous substrate proteins for this kinase within both soluble and particulate compartments of pancreatic acinar cells. Interactions such as these may provide clues for the understanding of the mechanisms of action of TPA and thus further our knowledge of Ca\(^{2+}\) as a modulator of exocrine pancreatic function.

MATERIALS AND METHODS

Chemicals. TPA, \(\beta\)-phorbol, phosphatidylserine (bovine brain), lysine-rich histone, trypsin inhibitor (type I-S), carbamylcholine, 2-amylose (type VIII-A), aprotinin, phenylmethylsulfonyl fluoride, and benzamidine were from Sigma Chemical Co., St. Louis, MO. Purified collagenase (low dextrin type) and chymotrypsin were obtained from Worthington Biochemicals, Freehold, N.J. MEM amino acid supplement was prepared using Grand Island Biological Co., Grand Island, N.Y. (\(\gamma\)-32P)ATP was prepared using the ProMega Biotech Kit.

Preparation of Isolated Pancreatic Acini. Adult male Wistar rats (200 to 250 g) were killed by decapitation. The pancreas was rapidly removed, and isolated pancreatic acini were prepared as described previously (28) by incubation for 50 min at 37\(^\circ\)C in a buffer containing 25 mM HEPES, pH 7.35 (103 mM NaCl, 4.78 mM KCl, 1.16 mM MgSO\(_4\), 2.0 mM CaCl\(_2\), 1.16 mM KH\(_2\)PO\(_4\), 20 \(\mu\)g trypsin inhibitor/ml, 14 mm glucose, and 1% MEM amino acid supplement) equilibrated with 95% O\(_2\)/5% CO\(_2\), containing MEM amino acid supplement, 0.01% soybean trypsin inhibitor, collagenase (70 to 90 units/ml), chymotrypsin (10 to 15 \(\mu\)g/ml), and BSA (2 mg/ml). Acini were dissociated by passage through polypropylene pipets of decreasing orifice diameters and filtered through nylon mesh. Thereafter, acini were resuspended in 10 mM HEPES-Ringer, pH 7.35, containing 1% BSA and placed onto a layer of 4% BSA. The cells were recovered by centrifugation and diluted to 2 mg/ml in HEPES-Ringer containing 0.1% BSA until experimentation. Cells prepared by this method were >95% viable (trypan blue exclusion). A population of pancreatic acini were incubated, and simultaneous measurements were made: (a) secretory activity was determined (28); and (b) distribution of PL/Ca-PK activity and endogenous substrate proteins for this kinase were examined either with or without the presence of phorbol ester at...
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Various concentrations and various times.

**Determination of Amylase and Protein.** Amylase was measured by the method of Jung, using Procion Yellow-coupled starch as substrate (10). Protein was determined by the method of Bradford (1).

**Fractionation of Pancreatic Acini.** Pancreatic acini were washed in 20 mM Tris-Cl, pH 7.5 (determined at room temperature), subsequently centrifuged, and resuspended in the same buffer, containing aprotinin (100 \(\mu\)g/ml), 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, and 50 mM 2-mercaptoethanol (Solution A). The cells were sonicated, and soluble PL/Ca-PK activity was rapidly recovered by centrifugation in an Eppendorf microfuge (15,000 rpm for 10 min). The pellet was washed once by resuspension in Solution A and centrifuged once more. The final membrane pellet was resuspended in Solution A, and PL/Ca-PK activity was recovered by treatment with 1% (v/v) NP40 and 10 mM EGTA in Solution A for 60 min at 4°C. Deionization and partial removal of detergent were accomplished by passing the suspension over a Sephadex G-25 column (Pharmacia Fine Chemicals, Inc., Piscataway, NJ) prior to assay.

**Protein Kinase Assay.** PL/Ca-PK activity was measured as described previously (31). The assay system (0.2 ml) contained: 20 mM piperazine-N,N' -bis(2-ethanesulfonic acid), pH 6.5; l-lysine-rich histone, 40 \(\mu\)g; MgCl\(_2\), 10 mM; phosphatidylserine, 5 \(\mu\)g; EGTA, 25 mM; with or without CaCl\(_2\), 0.5 mM; and 30 \(\mu\)g of sample protein per assay tube. The reaction was initiated by addition of \((\gamma\text{-}^{32}\text{P})\text{ATP, 1 nmol, containing 0.5 to 1.0 \(\times\) }10^6\text{ cpm and carried out at 30°C for 5 min. Protein kinase activity stimulated by Ca}\(^{2+}\) is reported or used for calculations.

**Phosphorylation of Endogenous Proteins.** Phosphorylation of endogenous substrate proteins was carried out in a reaction mixture (0.2 ml) containing: 25 mM Tris-Cl, pH 7.5; 10 mM MgCl\(_2\); 25 \(\mu\)M EGTA; sample protein (150 \(\mu\)g); 20 \(\mu\)M \((\gamma\text{-}^{32}\text{P})\text{ATP, containing 4 \(\times\) }10^6\text{ cpm; in the presence or absence of CaCl}_2\); phosphatidylserine (5 \(\mu\)g), and calmodulin (10 \(\mu\)M). Incubations were carried out at 30°C for 5 min and terminated by the addition of 0.1 ml of stop solution (30 mM Tris-Cl, pH 7.8, 9% SDS; 15% (v/v) glycerol; 0.05% bromophenol blue), followed by placing in boiling water bath for 3 min. Mercaptoethanol (20 \(\mu\)l) was added to each tube, and samples were kept overnight at 4°C.

**SDS-PAGE and Autoradiography.** SDS-PAGE of the phosphorylated samples was carried out as described previously (31) using 10% acrylamide separating gels and 5% stacking gels, both containing 0.1% SDS. Following electrophoresis, gels were stained with Coomassie Brilliant Blue, subsequently destained, dried under vacuum, and exposed to Kodak XRP-1 film for 1 to 6 days with the aid of Cronex Hi-Plus intensifying screens (Dupont). The amount of \(^{32}\text{P}\) incorporated into substrate proteins from each gel was quantitated using a scanning densitometer (Model 910; E-C Apparatus Corp.) with the area of individual phosphoprotein peaks measured in triplicate densitometric tracings using a quantitative image analysis system (Carl Zeiss, Inc.).

**RESULTS**

Effects of TPA treatment upon distribution of PL/Ca-PK activity from the soluble to the membrane compartment of pancreatic acinar cells were initially examined. Total PL/Ca-PK activity (soluble plus particulate) of control and TPA-treated acini remained unchanged for the duration of the experiment (Chart 1). In control acini, the activity of this kinase was evenly distributed between the 2 subcellular compartments. TPA treatment resulted in a marked increase in the amount of membrane-associated PL/Ca-PK activity, with a corresponding drop in activity of this enzyme in the soluble fraction (Chart 1). Actual values for control soluble and particulate were 40.83 ± 3.41 and 22.25 ± 2.88 (SE) pmol/min/mg, respectively, while values for TPA-treated cells were 10.67 ± 0.971 and 64.17 ± 6.39 pmol/min/mg. Recovery of total assayable PL/Ca-PK activity from the particulate fraction required extraction with EGTA (10 mM) and the detergent NP40, followed by buffer exchange. Membrane-associated PL/Ca-PK recovered in this way was sharply elevated in TPA-treated acini, accounting for the decrease noted in soluble kinase activity (Chart 1). It was also possible to demonstrate by partial purification of the extracted enzyme, using DEAE-cellulose, that TPA treatment resulted in elevating membrane-associated PL/Ca-PK activity above control (data not shown).

In further experiments, phospholipid/Ca\(^{2+}\)-dependent phosphorylation of endogenous proteins was examined in soluble and membrane fractions from control and TPA-treated acini. SDS-PAGE/autoradiography revealed the disappearance of at least 2 proteins (approximate \(M, 22,000\) and 18,000) phosphorylated in a phospholipid/Ca\(^{2+}\)-dependent manner from the soluble fraction of phorbol ester-treated acini (Fig. 1; compare Lanes 4 and 9). Concurrently, it was possible to demonstrate by partial purification of the extracted enzyme, using DEAE-cellulose, that TPA treatment resulted in elevating membrane-associated PL/Ca-PK activity above control (data not shown).

In conclusion, the results presented here demonstrate that TPA-induced translocation of both endogenous substrate proteins was accompanied by a concomitant increase in PL/Ca-PK activity in the particulate compartment of the acinar cell membrane site.
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SOLUBLE FRACTION

Fig. 1. Autoradiograph showing phosphorylation of endogenous proteins from the soluble fraction of control or TPA-treated acini. Acini were incubated for 30 min at 37°C in either the absence or the presence of TPA (10^-6 M). Cell fractionation, incubation conditions for phosphorylation, and subsequent SDS-PAGE/autoradiography were as described in "Materials and Methods." Additions were made as indicated and consisted of CaCl2 (Ca) (75 μM), phosphatidylserine (PS) (5 μg), and calmodulin (CaM) (10 μg), and 40 μg of protein/lane were charged for SDS-PAGE. Results are typical of 5 separate experiments. kD, molecular weight in thousands.

with initial detection as early as 5 min and sustained up to 30 min following exposure to the phorbol ester (Chart 3).

Correlative studies were conducted to determine the effect of TPA treatment on amylase secretion by intact pancreatic acini. As shown in Chart 4, a 2- to 2.5-fold increase in amylase release was noted in acini treated with TPA (10^-6 M) at a time (30 min) when maximum translocation of PL/Ca-PK and alteration in the location of the 2 substrate proteins was noted (Chart 3). This effect of TPA was comparable to the secretory response to these acini to the muscarinic agonist carbachol (Chart 4). Neither the nonesterified, non-tumor-promoting derivative β-phorbol nor the vehicle dimethyl sulfoxide had any stimulatory effect upon amylase secretion (Chart 4).

To further investigate the altered phosphorylation of soluble and particulate protein by PL/Ca-PK in phorbol ester-treated acinar cells, membranes from untreated cells were partially solubilized using the detergent NP40. Protein phosphorylation by endogenous PL/Ca-PK was stimulated by the addition of calcium and phosphatidylserine. Treatment of membranes with detergent was found to mimic the effect of TPA exposure (Fig. 3), with increased phosphorylation of the M, 22,000 and M, 18,000 proteins, as well as other minor bands not reproducibly phosphorylated.

DISCUSSION

In the present studies, we have demonstrated that treatment of rat pancreatic acini with minute quantities of biologically active phorbol ester (TPA) leads to a sustained elevation of PL/Ca-PK activity at a membrane site. These results are consistent with recent evidence (6, 14) suggesting that rapid association of PL/Ca-PK with the plasma membrane may be an early event in mediating some of the biological effects of phorbol esters.

A clear demonstration of TPA-dependent movement of soluble PL/Ca-PK required concurrent demonstration of a quantitative increase in membrane-associated kinase activity from phorbol ester-treated acini relative to control membranes. We found that extraction of the membrane fraction with detergent and EGTA was a necessary prerequisite in assaying for PL/Ca-PK activity. Otherwise, basal histone kinase activity in the absence of added Ca2+ was substantially elevated, obscuring any stimulation in the presence of added Ca2+. Membrane fractions extracted in this way were further processed by passage over Sephadex G-25. This treatment served to: (a) dilute and partially remove detergent; and (b) aid in the elimination of endogenous Ca2+, thereby lowering basal activity which would interfere with the histone kinase assay (11). Quantitative distribution of PL/Ca-PK between soluble and particulate compartments was accounted for, allowing direct comparison between control and phorbol ester-treated acini and clear demonstration of an increase in membrane-associated PL/Ca-PK in TPA-treated cells. It is possible that in situ PL/Ca-PK undergoes a conformational change upon translocation to the cell membrane during phorbol ester treatment or that TPA facilitates the entry of PL/Ca-PK into the membrane.
Chart 2. Effect of phorbol ester (TPA) concentration on the appearance of phosphorylated substrate proteins in the soluble fraction from TPA-treated acini. Acini were incubated for 30 min in the presence of various concentrations of TPA (as indicated). Fractionation, incubation conditions for phosphorylation, and subsequent SDS-PAGE/autoradiography were as described in "Materials and Methods." Substrate proteins indicated correspond to those demonstrated in Figs. 1 and 2. O, Control; M, 18,000 substrate protein; Δ, M, 22,000 substrate protein. Amounts of 32P incorporated were quantified by scanning densitometry as described in "Materials and Methods." Results presented are typical of 4 separate experiments.

where it becomes activated by binding to TPA directly or, alternatively, to diacylglycerol. This hypothesis is supported by in vitro studies (2) utilizing purified PL/Ca-PK which have suggested an alteration in enzyme conformation following TPA binding (a) by greatly increasing the affinity of the enzyme for Ca2+ and (b) by substituting for diacylglycerol. Our data further support the hypothesis that upon treatment with TPA a rapid and tight association between PL/Ca-PK and the acinar cell membrane occurs in such a manner as to allow the kinase to become an integral protein.

The finding that detergent treatment of membranes mimics the effect of phorbol ester upon calcium/phospholipid-dependent protein phosphorylation may provide additional information concerning mechanisms by which these tumor promoters effect changes in cellular activity. The present study presents results which indicate a differential phosphorylation of endogenous proteins by PL/Ca-PK in soluble and particulate compartments following treatment with TPA. It is possible that these alterations may reflect redistribution of substrate proteins within the cell following TPA treatment. An alternative interpretation, supported by the experiments using detergent-treated membranes (Fig. 3), is that preexisting membrane-associated substrate proteins become available to PL/Ca-PK (either native or transiently associated with the membrane). It is possible that TPA treatment results in conformational changes in kinase or substrate proteins or, in

Chart 3. Kinetics of TPA-induced alteration in the appearance of phosphorylated substrate proteins from the soluble fraction of TPA-treated pancreatic acini. Acini were incubated for various times (as indicated) in the absence (O, A) or presence (C, D) of TPA (10^{-6} M). Fractionation and incubation conditions for phosphorylation and subsequent SDS-PAGE/autoradiography were as described in "Materials and Methods." Substrate proteins indicated correspond to those demonstrated in Figs. 1 and 2. O, M, 18,000 substrate protein; Δ, M, 22,000 substrate protein. Amounts of 32P incorporated were quantified by scanning densitometry as described in "Materials and Methods." Results presented are typical of 3 separate experiments.

Chart 4. Effects of various agents on amylase secretion by pancreatic acini. The amount of amylase released was determined after 30 min exposure to various agents, as indicated. Aliquots of incubation mixture were removed at the appropriate time, acini were removed by rapid centrifugation, the incubation media were recovered, and amylase release was determined as described in "Materials and Methods." The pelleted cells were disrupted by sonication in water, and protein was determined. Values are the means of triplicate determinations and are representative of 8 independent experiments. Bars, SE; DMSO, dimethyl sulfoxide; U, units.
Calmodulin, which is responsible for the initiation of secretion; and (b) a second, expressed via PL/Ca-PK, which sustains the response. The present results suggest that this hypothesis may be extended to the control of pancreatic exocrine secretion. More detailed studies of the precise subcellular location of PL/Ca-PK and its endogenous substrate proteins following TPA treatment should further our understanding of the role that this kinase and Ca$^{2+}$ have in modulation of pancreatic exocrine function.

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REFERENCES


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