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LINOLEIC ACID IS A POTENT ACTIVATOR OF PROTEIN KINASE C TYPE III- α ISOFORM IN PANCREATIC ACINAR CELLS; ITS ROLE IN AMYLASE SECRETION

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Linoleic acid, an unsaturated-long chain fatty acid, was found to maximally activate protein kinase C (PKC) more effectively than arachidonic or linolenic acid, while the saturated fatty acids palmitic or arachidic had no stimulatory effect. Treatment of intact pancreatic acinar cells with linoleic acid resulted in dose-dependent phosphorylation of endogenous substrate proteins for this kinase and simultaneously stimulated amylase secretion in a dose- and time-dependent fashion. During chromatographic separation of pancreas protein kinase C activity, utilizing hydroxylapatite (HTP), Type III-α PKC isoform was detected. These data are consistent with a role for PKC in the regulation of pancreatic exocrine secretion.

Calcium ion is well documented as a regulator of pancreas exocrine function (1,2). Recent work in our laboratory (3-5) has provided evidence that secretion by pancreatic acini may be modulated by Ca^{2+} through phospholipid-sensitive/Ca^{2+}-dependent protein kinase (C-kinase, PKC). Activation of PKC in intact cells results from the transient generation of diacylglycerol by stimulus-induced phosphatidylinositol turnover, which subsequently increases the affinity of this kinase for calcium ion (6,7). Several endogenous substrate proteins for this kinase have been identified in broken cell preparations from pancreas (4) and most recently one has been localized to the zymogen granule membrane (5), thus linking this kinase system with the secretory apparatus. The possibility exists that

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calcium-dependent phosphorylation of one or more substrate proteins could result in alterations of their functional capacity and therefore affect cellular activities, such as secretion (8,9).

McPhail et al, (10) postulated that unsaturated fatty acids may serve as second messengers by activating PKC either alone or in the combined presence of diacylglycerol or phosphatidylserine. Unsaturated fatty acids have been shown to serve a physiological role in phagocytic cells by stimulating the respiratory burst and directly activating the respiratory burst enzyme in disrupted cell preparations from macrophages (11-13). During stimulus-induced turnover of inositol phospholipids, diacylglycerol is present within the membrane only transiently, either returning to inositol phospholipid or being further degraded to arachidonic acid, a long chain unsaturated fatty acid. Taken together, these findings suggest that activation of PKC activity by unsaturated fatty acids may represent another mechanism for the modulation of this protein kinase *in vivo*.

The PKC gene has been recently cloned and belongs to a gene family (14). In brain there are 4 specific PKC isotypes: Type I, gamma; Type II, $beta_{1,2}$ (alternatively spliced forms); and Type II, alpha. The gene product of these isoforms has been characterized by hydroxylapatite (HTP) column chromatography (15). In addition, Sekiguchi *et al*. (16) have recently investigated the effects of unsaturated and saturated fatty acids on each of these isoforms.

In the present study, we examine the effects of unsaturated and saturated fatty acids upon activation of PKC phosphorylation of endogenous proteins and amylase secretion by intact pancreatic acini in an attempt to investigate a potential relationship between this protein kinase system and regulation of exocrine pancreatic function. To further our understanding of the role this kinase system plays in exocrine secretion, we also undertook studies to examine which specific PKC isoform was present.

EXPERIMENTAL PROCEDURES

Materials

Phosphatidylserine (bovine brain), fatty acids, lysine-rich histone (Type III-S, corresponding to histone H_1), trypsin inhibitor and marker proteins used for Mr determination of endogenous substrate proteins in SDS-PAGE (phosphorylase B, 94kD; bovine serum albumin, 68kD; ovalbumin, 43kD; α -chymotrypsinogen, 25kD; soybean trypsin inhibitor, 21kD; and cytochrome C, 13kD) were from Sigma. γ-^{32}P -ATP was synthesized using the Promega Biotech Kit.

Preparation of C-kinase. C-kinase was purified from rat pancreas as described by Wooten *et al*. (17) and used as the source of enzyme throughout the study. Separation of PKC isotypes was achieved using hydroxylapatite column chromatography as previously described (15).

Preparation of Isolated Pancreatic Acini. Rats (fasted overnight) were killed, the pancreas removed and isolated pancreatic acini prepared

by the method described by Williams et al. (18). Cells prepared by this method were >95% viable (Trypan Blue exclusion). Isolated acini were incubated with various saturated or unsaturated fatty acids as indicated, and secretory activity was determined as previously described (3, 18).

Determination of Amylase and Protein: Amylase was measured by the method of Jung (19) using Procion Yellow dye coupled to starch as a substrate. Protein was determined by the method of Bradford (20).

Protein Kinase Assay: PKC activity was measured as previously described (17,21), substituting Pipes (pH 6.5) for Tris-Cl, (pH 7.5) as the assay buffer. The assay system (0.2 ml) contained Pipes, 20mM; lysine rich histone, 40 µg; $MgCl_2$, 10mM; phosphatidylserine, 5 µg; EGTA, 25 µM; with or without $CaCl_2$, 0.5mM; and 10-50 µg sample protein. The reaction was initiated by the addition of γ -^{32}P -ATP, 1nmol, containing 0.5-1.0 x 10^6cpm, and carried out at 30°C for 5 min. Protein kinase activity stimulated by Ca^{2+}/fatty acid is reported or used for calculations.

Phosphorylation of Endogenous Proteins: Broken cell preparations of pancreatic acini were prepared by sonication in 20mM Tris/Cl, pH 7.5, 50mM 2-mercaptoethanol, 1mM benzamidine, and 100 µg/ml aprotinin. Phosphorylation of endogenous proteins was carried out in a reaction mixture (0.2ml) containing 25mM Tris/Cl, pH 7.5, 10mM $MgCl_2$, 25 µM EGTA, sample protein (70-120µg), 20µM γ-^{32}P -ATP, containing about 4 x 10^7 cpm, in the presence of varying concentrations of fatty acids. Incubations were carried out at 30°C for 3 min, and terminated by the addition of 0.1 ml of stop solution (30mM Tris/Cl, pH 7.8; 9% SDS; 15% glycerol; 0.05% bromophenol blue) followed by placing in a boiling water bath for 3 min. 2-mercaptoethanol (20 µl) were added to each tube and samples kept overnight at 4°C.

SDS-PAGE and Autoradiography: SDS-PAGE of the phosphorylated samples was carried out as previously described (21). Following electrophoresis, gels were stained with Coomassie brillant blue, subsequently destained, dried under vacuum and exposed to Kodak XRP-1 film for 1-6 days with the aid of Cronex Hi-Plus intensifying screens (DuPont). The amount of ^{32}P incorporated into substrate proteins was quantitated using a scanning densitometer (Model 910, E-C Apparauts Corp.). The area of individual phosphoprotein peaks was measured above background in densitometric tracings using a quantitative image analysis system (MOP-1; Carl Zeiss, Inc.).

RESULTS AND DISCUSSION

Effects of unsaturated and saturated fatty acids upon activity of PKC (Table I) were examined. In the presence of calcium, linoleic acid was equipotent to phosphatidylserine in activating PKC while the other unsaturated fatty acids (arachidonic and linolenic) tested were slightly less effective (Table I). Saturated fatty acids (palmitic and arachidic) were markedly less potent in activating PKC. We were unable to detect an additive effect upon enzyme activation in the presence of phosphatidylserine and any of the fatty acids tested. In addition, stimulation of enzyme activity by unsaturated fatty acids required the presence of calcium ion.

Since linoleic acid could effectively activate PKC in vitro, we chose to compare effects of treatment with this fatty acid upon endogenous substrate phosphorylation. Broken cell preparations of pancreatic acini

TABLE I
Effects of saturated and unsaturated fatty acids on activation of protein kinase C

	Fatty Acid	Protein Kinase Activity (pmol/min/mg)	% Activation
Unsaturated	Linoleic Acid	969.90 ± 35	101%
	Arachidonic Acid	679.39 ± 22	71%
	Linolenic Acid	640.15 ± 41	67%
Saturated	Palmitic	150.55 ± 4	16%
	Arachidic	380.90 ± 63	40%
	Phosphatidylserine	958.80 ± 53	100%

Activation of protein kinase C in various free fatty acids. Protein kinase activity was measured as described in Methods. Fatty acids (100 μM) and phosphatidylseine (5 μg) were dispersed into Tris-HCl at pH 7.5 by vigorous vortex - mixing for 1 min, followed by sonication for 5 min at 4°C. Data represent increase (pmol/min/mg) in protein kinase C activity in the presence of 500 μM $CaCl_2$ and the indicated fatty acid minus background (EGTA alone). The data are means ± S.E.M. from one experiment (triplicate incubations) and are representative of four other independent experiments. The % activation represents comparison of maximal activation obtained for PKC in the presence of phosphatidylserine/$CaCl_2$ (100%) versus that obtained with the various fatty acids/$CaCl_2$.

were incubated in the presence of linoleic acid at various concentrations plus γ-^{32}P ATP. Such an experiment revealed enhanced phosphorylation of several previously described substrate proteins (94kD, 56kD, 30kD, 22kD, 18kD, and 15kD) for PKC (4,20; Fig. 1). Densitometric analysis of autoradiograms revealed that phosphorylation of these proteins was enhanced from 3 to 15 fold over a 1-500μM concentration range. Endogenous substrate phosphorylation was not enhanced by a saturated fatty acid, palmitic acid (data not shown).

Intact pancreatic acini was incubated in the presence of various fatty acids and exocrine secretory activity assessed. The unsaturated fatty acids (linoleic, arachidonic and linolenic) were consistently effective in stimulating amylase secretion (Fig. 2). Linoleic acid was by far the most potent, followed by arachidonic and linolenic. The saturated fatty acids were either marginally effective or completely ineffective as secretory stimuli. The stimulatory effect of linoleic acid upon amylase release was rapid (noted within 15 min; data not shown) and dose-dependent (Fig. 3).

Chromatographic separation of PKC isoforms utilizing HTP (15) revealed the presence of an eluting peak of activity ~ 110mM [KPO_4] (Fig. 4). Utilizing rat brain as a chromatographic reference Type I = gamma (40 - 60mM KPO_4); Type II = beta (70 - 90mM, KPO_4); Type III = alpha (100 - 150mM, KPO_4), we conclude that the pancreas PKC isotype has an elution

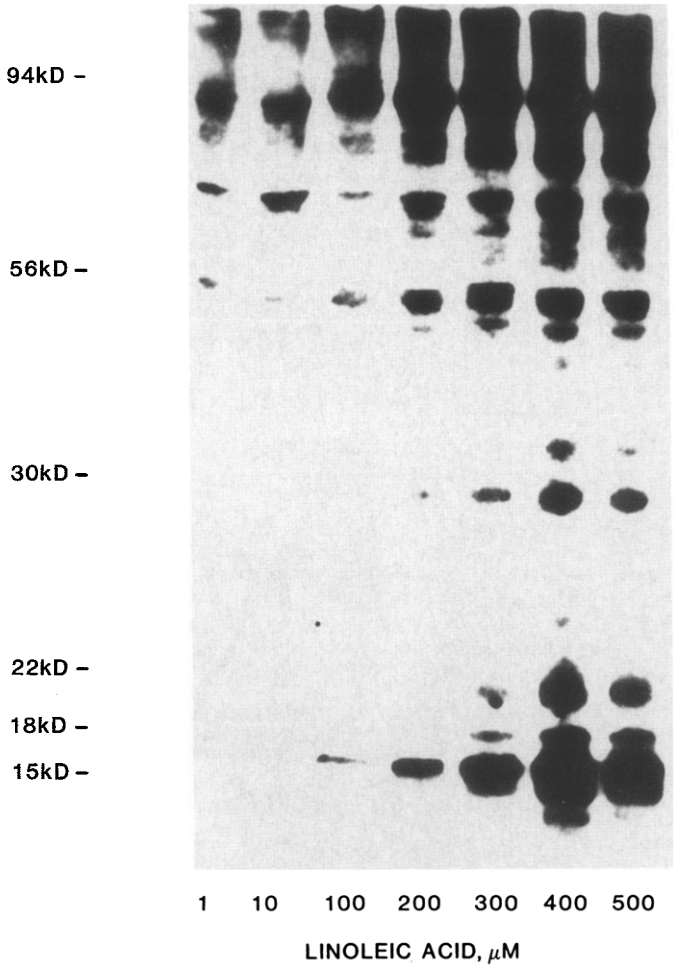
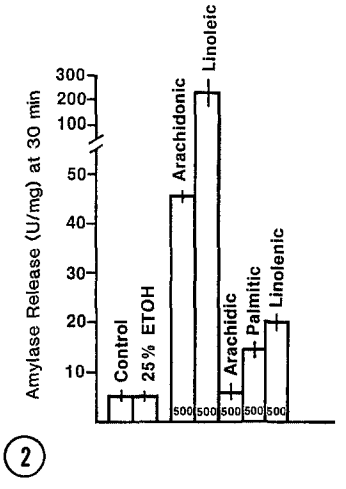


Figure 1. Autoradiograph showing the effects of linoleic acid on endogenous substrate phosphorylation. Broken cell preparations of pancreatic acini were incubated in the presence of various concentrations of linoleic acid and γ -^{32}P -ATP, as indicated. Conditions for phosphorylation and subsequent SDS-PAGE/autoradiography were as described in Methods. Equivalent amounts of protein per lane were charged for SDS-PAGE. Results are typical of two other independent experiments.

pattern coincident with brain Type III = alpha PKC isoform (15,22). These results are consistent with the findings that linoleic acid activates Type III - alpha PKC to a far greater degree (16). In addition, this is one of the first studies to demonstrate localization of a PKC - isotype within a specific organ system, characterize its substrate proteins, and relate these findings to a specific physiological function.

Recent studies have shown that unsaturated fatty acids can directly stimulate a respiratory burst in phagocytic cells (11,13). Several receptor-mediated responses, including agonists such as muscarinic cholinergic, thrombin, and histamine, stimulate phosphatatidylinositol



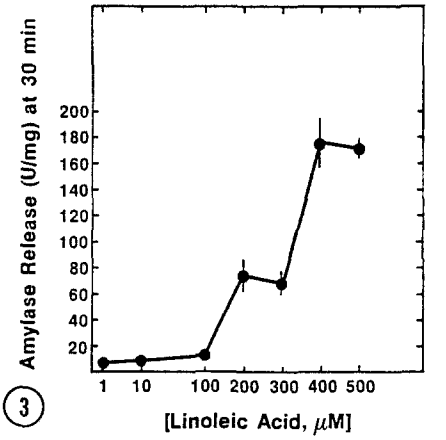


Figure 2. Effect of saturated and unsaturated fatty acids on amylase release from pancreatic acini. Acini were incubated for 30 min in the presence of various fatty acids (500 μM) as indicated, either saturated (palmitic and arachidic) or unsaturated (arachidonic, linoleic, linolenic). After incubation, the acini was removed by rapid centrifugation, amylase released into the medium and cellular protein determined. Results are typical of 3 experiments and represent the means of triplicate determinations ± S.E.M.

Figure 3. Effect of unsaturated fatty acid (linoleic acid) treatment on amylase release from pancreatic acini. Acini were incubated for 30 min in the presence of various concentrations of linoleic acid, as indicated. After incubation, the acini were removed by rapid centrifugation, amylase released into the medium and cellular protein was determined. Results given represent the means of triplicate incubations (± S.E.M.) and are typical of two other independent experiments.

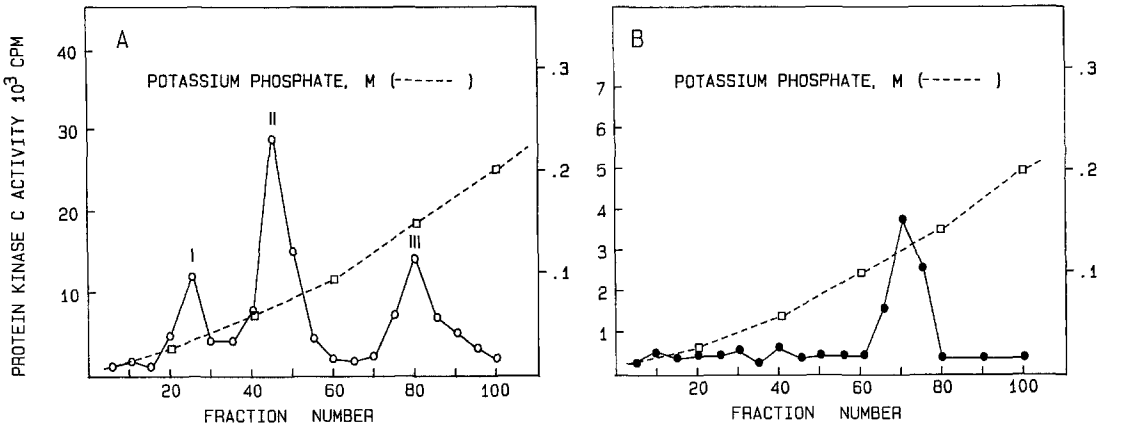


Figure 4. Chromatographic separation of PKC isoforms. Rat brain and pancreas PKC were isolated as described in Methods and further separated using HTP. Panel A: Rat Brain PKC isotype chromatographic separation, Peak I = Type I = γ; Peak II = Type II = β; Peak III = Type III α. Panel B: Pancreas PKC isotype chromatographic separation, Peak III = Type III = α.

turnover and release of unsaturated fatty acids after receptor binding (23-27). In addition, previous work (4,23) with broken cell preparations has shown that several defined substrate proteins for PKC exist in pancreatic acinar cells (94kD, 56kD, 30kD, 22kD, 18kD, and 15kD), and Wrenn (5) has recently shown that an 18kD protein phosphorylated in a calcium- and phospholipid-dependent manner is localized to the zymogen granule membrane. Taken together, these studies demonstrate that protein phosphorylation via a protein kinase C - mediated pathway may play a role in stimulus-secretion coupling and exocrine pancreatic function.

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