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Research Article

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Diminished Agonist-stimulated Inositol Trisphosphate Generation Blocks Stimulus–Secretion Coupling in Mouse Pancreatic Acini during Diet-induced Experimental Pancreatitis

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Abstract

Young female mice fed a choline-deficient, ethionine-supplemented (CDE) diet rapidly develop acute hemorrhagic pancreatitis. We have observed that pancreatic acini prepared from these mice are unable to secrete amylase in response to addition of the cholinergic agonist carbachol, although they retain the ability to secrete amylase in response to the Ca^{2+} ionophore A23187. The CDE diet does not alter the binding characteristics (K_d or the maximal number of binding sites) for muscarinic cholinergic receptors as tested using the antagonist [3H]N-methylscopolamine nor the competition for this binding by carbachol. Addition of carbachol to acini prepared from mice fed the CDE diet does not result in as marked an increase in cytosolic free Ca^{2+} levels as that noted in control samples (evaluated using quin2 fluorescence). These observations indicate that the CDE diet interferes with stimulus–secretion coupling in mouse pancreatic acini at a step subsequent to hormone–receptor binding and prior to Ca^{2+} release. This conclusion is confirmed by our finding that the hormone-stimulated generation of [3H]inositol phosphates (inositol trisphosphate, inositol bisphosphate, and inositol monophosphate) from acini labeled with [3H]myo-inositol is markedly reduced in acini prepared from mice fed the CDE diet. This reduction is not due to a decrease in phosphatidylinositol-4,5-bisphosphate. This communication represents the first report of a system in which a blockade of inositol phosphate generation can be related to a physiologic defect and pathologic lesion.

Introduction

Agonist-induced phosphatidylinositol hydrolysis, first observed in the cells of the exocrine pancreas, appears to be of widespread importance in stimulus–effect coupling in numerous systems (1–3). One of the most well-studied stimulus–secretion systems is that which involves hormone-stimulated secretion of digestive enzymes from the exocrine pancreas. Currently available data, obtained from studies of pancreatic acinar cells, indicate that agonist–receptor interaction leads to activation of a phospholi-

pase C, which cleaves phosphatidylinositol-4,5-bisphosphate (PIP_2)¹ into inositol trisphosphate (IP_3) and 1,2-diacylglycerol (4–7). IP_3 is then thought to induce a rise in the cytosolic free Ca^{2+} concentration by release of Ca^{2+} from storage pools in the endoplasmic reticulum (8). The rise in cytosolic Ca^{2+} (9, 10) triggers a series of steps ultimately leading to secretion (11, 12). This secretion may be due to the phosphorylation of certain cellular proteins by calmodulin-regulated protein kinases which are activated by the increased cytosolic Ca^{2+} levels (13). Concomitant with the production of IP_3 by phospholipase C is the generation of diacylglycerol, which has been reported to activate the Ca^{2+} -, phospholipid-dependent protein kinase (protein kinase C) in many systems including the pancreas (14, 15). Protein kinase C also phosphorylates cellular proteins which may represent additional intermediate steps in stimulus–secretion coupling (16–18).

In physiologic studies, Lombardi and his co-workers (19, 20) observed that feeding young female mice a choline-deficient, ethionine-containing (CDE) diet caused the rapid onset of acute hemorrhagic pancreatitis. Subsequently, it was noted that secretagogue-induced secretion of digestive enzymes was inhibited in CDE-treated mice although the content of these enzymes in the cells was actually increased (21, 22). These observations suggested that the CDE diet administration might interfere with the processes involved in stimulus–secretion coupling, and that this defect might be related to the development of this form of experimental pancreatitis.

In this article we report studies examining the effects of the CDE diet upon the various biochemical events that couple hormonal stimulation to digestive enzyme secretion in pancreatic acinar cells. The results of these studies indicate that the CDE diet inhibits the generation of IP_3 from PIP_2 . This biochemical lesion, which is the first reported to involve blockade of IP_3 generation, appears to be the event that ultimately leads to the development of acute hemorrhagic pancreatic necrosis in this model.

Methods

Animals. Female mice (10–15 g) were fasted overnight and fed 3 g per animal of either a CDE diet (19) or standard laboratory diet (control) over a 24-h period. After an additional 24-h fast, mice were killed by cervical dislocation and dispersed pancreatic acini were prepared by collagenase treatment and gentle shearing as previously described (10). Acini

The results of the study were reported in part at the 1985 meeting of the American Gastroenterological Association and have been published as an abstract (1985. *Gastroenterology*. 88:1544).

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1. **Abbreviations used in this paper:** CCK, cholecystokinin; CDE, choline-deficient 0.5% ethionine-containing (diet); IP_3 , IP_2 , and IP_1 , inositol trisphosphate, bisphosphate, and monophosphate; [3H]NMS, [3H]N-methylscopolamine; OAG, 1-oleoyl-2-acetyl-glycerol; PI, phosphatidylinositol; PIP, phosphatidyl-4-phosphate; PIP_2 , phosphatidylinositol-4,5-bisphosphate; TPA, 12-O-tetradecanoylphorbol-13-acetate.

were resuspended in Hepes-Ringer buffer (pH 7.4) at 37°C, which was composed of 115 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 1 mM Na₂HPO₄, 10 mM Hepes, 15 mM glucose, 1.26 mM CaCl₂, 0.1% bovine serum albumin (BSA), 0.01% soybean trypsin inhibitor, and Eagle's basal amino acids. The buffer was saturated with O₂. Viability of acini was >95% as assessed by trypan blue exclusion.

[³H]N-methylscopolamine ([³H]NMS) binding. Aliquots of acini (~10 μg of DNA) were resuspended in Hepes-Ringer buffer containing varying concentrations of [³H]NMS in a total volume of 0.5 ml. Incubations were continued for 45 min under an atmosphere of 95% O₂ and 5% CO₂ in a shaking water bath at 37°C. Incubations were terminated by the addition of 15 ml of ice cold phosphate-buffered saline and the samples were subjected to vacuum filtration through Whatman GF/C filters (Whatman Inc., Clifton, NJ). The filters were placed into 10 ml of Beckman Readi-Solv HP (Beckman Instruments, Inc., Fullerton, CA) and allowed to sit overnight, and the radioactivity was counted in a Packard Tri-Carb liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, IL). Nonspecific binding was defined as the bound radioactivity noted in the presence of 10⁻⁵ M atropine and was typically <10% of total binding in the presence of 0.5 nM [³H]NMS. Specific binding was defined as the difference between total binding, measured in the absence of atropine, and nonspecific binding measured in the presence of 10⁻⁵ M atropine. The K_i values of agonist competition analyses were calculated by the method of Cheng and Prusoff (23).

Quin2 fluorescence. Acini were resuspended in Hepes-Ringer buffer and incubated with 20 or 50 μM quin2/acetoxymethylester for 20 min. They were then washed twice with Hepes-Ringer buffer and the quin2 fluorescence was determined as previously described (10).

Measurement of inositol phosphates. The cellular phosphatidylinositol (PI) pool was labeled by incubating acini in Hepes-Ringer buffer plus 1 mM MnCl₂ and 100 μCi/ml of [³H]myoinositol for 1 h at 37°C under an atmosphere of 95% O₂ and 5% CO₂ with constant shaking. The acini were then washed once with buffer and resuspended in Hepes-Ringer buffer containing 10 mM LiCl without BSA. After a 10-min preincubation, aliquots were placed into tubes with or without secretagogues. After suitable incubation periods, 2 ml of cold 0.6 N perchloric acid was added and the resulting samples were allowed to precipitate for 30 min. After centrifugation, the supernatant was neutralized to approximately pH 8 by addition of a neutralizing buffer (0.5 M KOH, 9 mM Na₂B₄O₇, and 1.9 mM EDTA) and the inositol phosphates separated by ion-exchange chromatography on Dowex-1×8, formate form (7, 24). Elution fractions were counted in Aquasol cocktail (New England Nuclear, Boston, MA) in the gel state. Results for inositol phosphate production are expressed as a percentage of the initial radioactivity in each inositol phosphate fraction at *t* = 0. Incorporation of radioactivity into the cellular lipids was determined by extraction of the perchloric acid pellets into the organic phase of a system consisting of chloroform/methanol (2:1) and 2 M KCl.

PI fractionation. Control or CDE-treated acini were loaded with [³H]myoinositol (30 μCi/ml) for 2 h. The acini were pelleted by centrifugation at 11,000 *g* for 10 s. The pellet was placed into 5 ml of ice-cold chloroform-methanol (2:1) and extracted against 5 ml of 1 N HCl. An additional 5 ml of chloroform was added and the organic layer was washed twice with cold 1 N HCl. The final organic layer was dried under nitrogen, washed twice with benzene to remove any remaining water as the azeotrope, and redissolved in chloroform. An aliquot was spotted onto a thin-layer chromatography plate of silica gel H with 1% potassium oxalate and developed in a phase of CHCl₃/CH₃OH/4 N NH₄OH (9:7:2) (25). PI, phosphatidylinositol-4-phosphate (PIP), and PIP₂ were located by radioinert standards and scraped from the plate, and the labeled compounds were quantitated by liquid scintillation counting.

The fraction of total phospholipid that is PIP₂ was determined by measurement of inorganic phosphorous. Six pancreata from control or CDE-treated mice were pooled and homogenized with a Polytron (Brinkmann Instruments, Westbury, NY), extracted by the method of Schacht (26), and PIP₂ separated by thin-layer chromatography as described above. PIP₂ was extracted from the silica gel with the running solvent system. The PIP₂ extract and aliquots of the total lipid extract

were assayed for inorganic phosphorous after digestion with perchloric acid by a modification of the method of Sanui (27).

Amylase secretion. Amylase activity was measured by the method of Bernfeld (28) using soluble starch as the substrate. Secretion is reported as the net stimulated increase of amylase activity in the medium as a percent of total amylase (10). DNA was measured by the method of LaBarca and Paigen (29).

Analysis of data. Values reported are the mean±standard error for the number of separate experiments indicated. Groups were compared statistically by Student's *t* test for unpaired observations, with the acceptance criteria of *P* < 0.05.

Materials. Female CD-1 mice (12–15 g) were purchased from Charles River Breeding Labs, Boston, MA; EGTA, carbamylcholine HCl, and Hepes were purchased from Sigma Chemical Co., St. Louis, MO; D,L-ethionine was purchased from United States Biochemical Corp., Cleveland, OH; Quin2/acetoxymethylester was purchased from Lancaster Synthesis (United Kingdom); collagenase (CLSPA) and soybean trypsin inhibitor were purchased from Cooper Diagnostics (Freehold, NJ), Eagle's Basal Amino Acids (100 ×) from Gibco (Grand Island, NY), Dowex 1×8 (formate form) from Bio-Rad Laboratories (Richmond, CA), and BSA from Miles Laboratories (Elkhart, IN); PI, PIP, and PIP₂ were purchased from Sigma Chemical Co.; silica gel H thin-layer plates impregnated with 1% potassium oxalate were purchased from Analtech, Inc., Newark, DE; [³H]NMS (84.8 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Myo-2-³H(*N*)-inositol (14 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Cholecystokinin (CCK)-octapeptide was a gift from Squibb Laboratories, Princeton, NJ. All other chemicals were from common sources.

Results

Amylase secretion. Pancreatic acini that were prepared from mice fed the control diet secreted amylase in response to both carbachol and the Ca²⁺ ionophore, A23187, with maximal responses that were roughly equivalent (Table I). In contrast, amylase secretion from acini prepared from mice fed the CDE diet was markedly inhibited (*P* < 0.001) after stimulation with either carbachol (Table I) or CCK-octapeptide (not shown). Dose-response studies evaluating amylase secretion in acini prepared from mice fed the CDE diet showed no secretory response to any carbachol dose tested (Fig. 1). In contrast, the Ca²⁺ ionophore, A23187, stimulated amylase secretion from acini prepared from mice on the CDE diet. No difference was detected when A23187-induced amylase secretion was compared between acini prepared from CDE or control diet-fed animals (Table I).

Table I. Effect of CDE Diet on Stimulated Amylase Secretion from Mouse Pancreatic Acini

Agent	Amylase	
	Control	CDE
μM	% total secreted/10 min	% total secreted/10 min
Carbachol		
1	7.9±1.5 (4)	0.13±0.3 (10)*
A23187:		
1	3.5±1.2 (3)	4.1±0.8 (16)
10	5.6±1.1 (3)	5.9±0.8 (15)

Net stimulated amylase secretion (expressed as a percentage of total over 10 min) in control and CDE mouse pancreatic acini is shown. Values are mean±SE for the number of separate experiments noted in parentheses.

* *P* < 0.001 by Student's *t* test, compared with control.

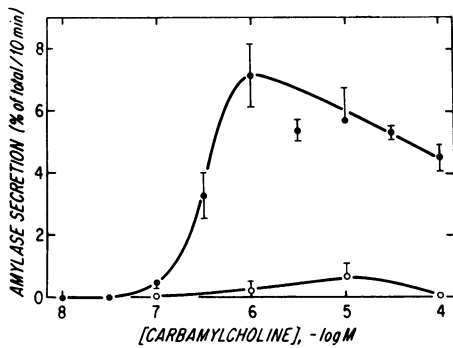


Figure 1. Amylase secretion (net stimulated as a percentage of the total amylase released/10 min) from control (●) and CDE (○) mouse pancreatic acini in response to carbachol. Points represent mean±SE for at least three experiments.

[³H]NMS binding. To determine whether the inability of acini prepared from CDE diet-fed mice to respond to carbachol resulted from an abnormality in the cholinergic receptor, [³H]NMS was used to evaluate muscarinic cholinergic binding characteristics in intact mouse pancreatic acini. Scatchard analysis of data obtained in binding studies indicated the presence of a high-affinity [³H]NMS-binding site with parameters similar to those observed in rat acini (30, 31). No difference in either the apparent dissociation constant (K_d) or number of binding sites (B_{max}) for the antagonist was observed when acini prepared from animals fed the CDE diet were compared with those fed the control diet (Table II). Competition for [³H]NMS binding by an agonist (carbachol) was also not altered in CDE-treated acini (Table II). The agonist displacement curves for control and CDE animals were superimposable (not shown). This indicates that the loss of response to carbachol in CDE-treated animals is not attributable to a loss in muscarinic cholinergic receptor function, but to some subsequent step in stimulus–secretion coupling.

Quin2 fluorescence. The resting cytosolic free Ca^{2+} concentrations determined by measuring quin2 fluorescence, in acini prepared from control and CDE diet-fed mice were not different, being 82 ± 8 and 94 ± 9 nM, respectively ($P > 0.05$). The agonist-stimulated increase in cytosolic Ca^{2+} concentration was measured by monitoring the increase in quin2 fluorescence that followed the addition of a maximally stimulating dose of carbachol (10 μ M) to acini prepared from either control or CDE diet-fed mice. This maximal stimulated increase in cytosolic Ca^{2+} was much less in acini prepared from CDE diet-fed mice (128 ± 11 nM) when compared to acini from control animals (451 ± 58 nM) ($P < 0.001$). This indicates that the CDE diet does not alter resting Ca^{2+} levels but that it induces a block in stimulus–secretion coupling that reduces the agonist-stimulated increase in cytosolic free Ca^{2+} levels.

The secretagogue-induced rise in cytosolic free Ca^{2+} was not, however, totally absent in acini prepared from CDE diet-fed animals. In the presence of a maximally stimulating dose of carbachol (10 μ M), the cytosolic free Ca^{2+} concentration rose by a value of 128 ± 11 nM to reach a level of 230 ± 16 nM in acini from CDE-treated mice. A similar small increase (99.5 ± 31 nM) in cytosolic free Ca^{2+} resulting in a Ca^{2+} level of 192 ± 36 nM could be elicited by adding a submaximally stimulating dose of carbachol (1 μ M) to acini prepared from control diet-fed mice (Fig. 2). Although comparable Ca^{2+} increases and peak levels

Table II. Effect of CDE Diet on Binding to Muscarinic Cholinergic Receptors

Ligand	Control	CDE
Antagonist		
B_{max} , pmol/mg DNA	2.46 ± 0.30	2.88 ± 0.62
K_d , nM	0.65 ± 0.12	0.75 ± 0.17
Agonist		
K_i , μ M	36 ± 15	49 ± 15

Mouse pancreatic acini were incubated with varying concentrations of [³H]NMS, as described in the text, to characterize antagonist binding to the muscarinic cholinergic receptor. Agonist binding was evaluated by measuring the inhibition of [³H]NMS (1 nM) binding induced by varying concentrations of carbachol and the K_i calculated as described by Cheng and Prusoff (23). Results represent mean±SE values from three or four separate experiments.

could be reached by using these different concentrations of agonist in acini from control and CDE diet mice, the secretion of amylase under these conditions was strikingly different. Amylase secretion from control acini was still 2.9 times that released from CDE acini (Fig. 2). This finding indicates that the lesion induced by the CDE diet is more complex than the mere interference with Ca^{2+} release and that another abnormality (see Discussion) may also be induced by the CDE diet.

Inositol phosphate production. Feeding the CDE diet does not appear to alter either the basal polyphosphatide or inositol phosphate metabolism. Mouse pancreatic acini incubated with [³H]myo-inositol incorporated radioactivity to a comparable extent whether prepared from control ($5,629 \pm 587$ dpm/ μ g DNA, $n = 4$) or CDE-treated ($5,540 \pm 1,307$ dpm/ μ g DNA, $n = 3$) mice. The level of PIP₂ (expressed as a percentage of total lipid phosphorous) was $0.16 \pm 0.03\%$ for the control pancreas and $0.15 \pm 0.1\%$ in CDE-treated pancreas (three separate determinations for each group). In addition, the labeling of PI and the polyphosphoinositides by [³H]myo-inositol was not different between control and CDE-treated acini (Table III). The resting levels of tritiated inositol trisphosphate, bisphosphate, and monophosphate ([³H]IP₃, [³H]IP₂, and [³H]IP₁) were not altered ($P > 0.05$) in acini prepared from mice fed the CDE diet when compared to control animals (see legend to Fig. 3).

Control mouse acini generated radiolabeled IP₃, IP₂, and IP₁ in response to either carbachol or caerulein stimulation (Fig. 3). Secretagogue-stimulated [³H]IP₃ production was rapid and dose-dependent. Maximal levels of [³H]IP₃ were observed within 5 s of secretagogue addition and the elevated levels of [³H]IP₃

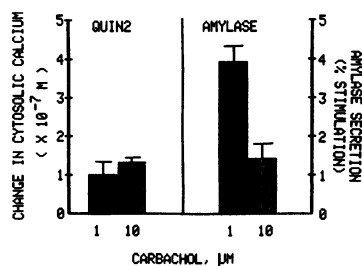


Figure 2. Change in cytosolic free Ca^{2+} levels, as determined by the quin2 fluorescence change (left), and amylase secretion as the net stimulated secretion, % of total/10 min (right). Control mouse pancreatic acini (solid bars) were treated with 1 μ M carbachol and CDE acini (striped bars)

were treated with 10 μ M carbachol to give equivalent quin2 responses. Values are mean±SE for between four and eight separate experiments.

Table III. Incorporation of [³H]Myoinositol into Pancreatic Acini Phospholipids

	Control	CDE
	% of total cpm	% of total cpm
PI	90.1±2.0	91.7±1.4
PIP	2.8±0.6	2.6±0.5
PIP ₂	1.8±0.06	1.6±0.2

Acini were labeled for 2 h with [³H]myoinositol (30 μCi/ml). The phospholipids were then extracted and separated by thin-layer chromatography as described in Methods. Results are expressed as the percent of the total CPM applied to the plate in each phosphoinositide fraction. Values are the mean±SE for three separate determinations.

remained constant for 5 min in the presence of 10 mM LiCl (32). Acini prepared from mice fed the CDE diet also produced [³H]IP₃ and [³H]IP₂ in response to carbachol stimulation, but at a much lower level than acini prepared from control diet-fed mice (Fig. 3). The levels of [³H]IP₃ and [³H]IP₂ measured in acini from animals fed the CDE diet were significantly less (*P* < 0.05) than those in acini from animals fed the control diet at all carbachol doses examined. [³H]IP₃ production in CDE-fed mouse acini was only 33% of that noted in acini from control diet-fed animals after the addition of 10⁻⁵ M carbachol, a dose that gave maximal amylase secretion from control acini. No production of [³H]IP₁ was detected in acini from CDE-fed mice in response to any concentration of carbachol tested. These results indicate that the CDE diet interferes with the generation

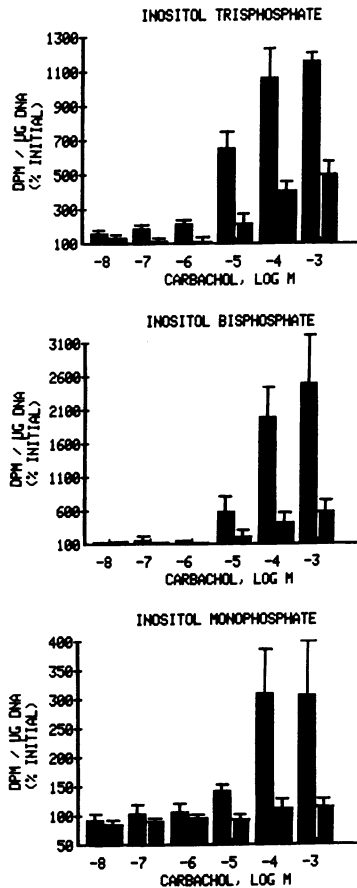


Figure 3. [³H]IP₃, [³H]IP₂, and [³H]IP₁ generation in dispersed mouse pancreatic acini in response to carbachol. Incubations were performed for 5 min in the presence of 10 mM LiCl. Results represent the level of each [³H]IP as a percentage of the initial disintegrations per minute in the incubation. Initial values (dpm/μg DNA) for [³H]IP₁, [³H]IP₂, and [³H]IP₃ were 1,341±146, 61±16, and 50±8 for control acini and 1,281±318, 42±20, and 29±9 for CDE-treated acini, respectively. Values represent the mean±SE for three separate experiments in each group. There are no statistical differences between the individual initial IP values for the two groups (*P* > 0.05).

of inositol phosphates in mouse pancreatic acini, and that this is not the result of a decrease in phospholipase C substrate (i.e., PIP₂) levels in the CDE-treated acini.

Protein kinase C activators. Mouse pancreatic acini secreted amylase in response to compounds that have been reported to mimic the action of diacylglycerol to stimulate protein kinase C activity: a phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and a synthetic diacylglycerol, 1-oleoyl-2-acetyl-glycerol (OAG). Maximal secretion to these compounds was, however, only ~40% of the maximal response observed to carbachol. Amylase secretion in response to maximally stimulating doses of these compounds was not different between control and CDE-treated mouse pancreatic acini (Table IV). These data are consistent with our hypothesis that the CDE diet-induced lesion blocks stimulus-secretion coupling at a step preceding the hydrolysis of the phosphoinositides, as demonstrated by the decreased inositol phosphate generation. Concomitant with this would be a decrease in the production of diacylglycerol. The finding that the secretory response is not different between the control and CDE-treated groups after stimulation with a phorbol ester or a synthetic diacylglycerol indicates that the primary block must precede the generation of endogenous diacylglycerol.

Discussion

The digestive enzyme content and number of zymogen granules in pancreatic acinar cells is increased within 24 h after young female mice are fed the CDE diet (21). Our previously reported biochemical and morphologic studies have indicated that this occurs because the CDE diet blocks digestive enzyme secretion while digestive enzyme synthesis and intracellular transport to the zymogen granule compartment continue unimpeded (21, 22). Prior studies, however, have not examined the mechanism by which the CDE diet interferes with secretion.

It is generally believed that the biochemical pathway that leads to digestive enzyme secretion by pancreatic acinar cells requires the secretagogue-induced elevation of cytosolic Ca²⁺ levels (11, 12). Our observation that the Ca²⁺ ionophore A23187 can induce secretion from acini made unresponsive to the muscarinic cholinergic secretagogue carbachol by the CDE diet (Table I) indicates that these acini retain the ability to secrete digestive enzymes once cytosolic Ca²⁺ levels are increased. This finding suggests that the CDE diet interferes with the process of secretagogue-induced elevation of cytosolic Ca²⁺. Support for this conclusion is provided by our studies that utilized the Ca²⁺-

Table IV. Stimulation of Amylase Secretion from Mouse Pancreatic Acini by Protein Kinase C Activators

Agent	Amylase	
	Control	CDE
	% total secreted/30 min	% total secreted/30 min
Carbachol, 1 μM	7.9±1.5 (4)	0.13±0.3 (10)
TPA, 1 μM	4.4±1.0 (9)	4.7±0.9 (10)
OAG, 40 ng/ml	3.5±0.7 (3)	4.9±0.7 (5)

Acini were prepared from control or CDE-diet fed mice. The acini were incubated for 30 min in the presence of a maximally stimulating dose of either agonist. Values represent the mean±SE for the number of separate experiments indicated in parentheses.

sensitive fluorescent probe quin2 to measure changes in the cytosolic free Ca^{2+} concentration after carbachol addition. Only a small change in the concentration of cytosolic Ca^{2+} followed addition of the secretagogue (Table III).

Failure of the secretagogue to elevate cytosolic Ca^{2+} levels and stimulate amylase secretion could indicate that the CDE diet had simply caused a loss of receptor function. To determine whether the CDE diet-induced loss of stimutable secretion was a receptor or postreceptor effect, we examined the effect of the diet on muscarinic cholinergic receptors by characterizing the binding of the radiolabeled antagonist [^3H]NMS to acini prepared from mice fed the control or CDE diet. No change in either the calculated number of NMS receptors (i.e., B_{max}) or dissociation constant for [^3H]NMS (i.e., K_d) was noted (Table II). In addition, there was no difference in the agonist competition for [^3H]NMS binding between control and CDE-treated acini (Table I). Thus, by exclusion, these findings indicate that the lesion induced by the CDE diet involves the process of stimulus-secretion coupling at a step subsequent to hormone-receptor binding and prior to Ca^{2+} mobilization.

Studies in the pancreas recently reported by Streb et al. (8), and supported by numerous experiments performed using other tissues (33), have shown that hormone-receptor occupancy leads to the hydrolysis of membrane phospholipids with the release of 1,4,5- IP_3 and that 1,4,5- IP_3 causes a rise in cytosolic Ca^{2+} levels by mobilizing Ca^{2+} from stores within the endoplasmic reticulum. To further define the lesion induced by the CDE diet, we performed studies to evaluate the generation of inositol phosphates in acini prepared from animals fed the CDE or control diets.

The CDE diet did not significantly alter the observed resting levels of [^3H]IP $_3$, [^3H]IP $_2$, and [^3H]IP $_1$, in acini preloaded with [^3H]myoinositol, when compared to control acini. However, secretagogue-induced increases in these labeled inositol phosphates were severely inhibited by the CDE diet. The rises in [^3H]IP $_3$ and [^3H]IP $_2$ were markedly reduced while that of [^3H]IP $_1$ was prevented by the CDE diet (Fig. 3). These findings indicate that the inability of the muscarinic cholinergic agonist to increase cytosolic Ca^{2+} and stimulate amylase secretion from acini prepared from mice fed the CDE diet is a consequence of the inability of carbachol to stimulate phospholipase C hydrolysis of PIP $_2$ and generation of IP $_3$ in these acini.²

Hydrolysis of PIP $_2$ by phospholipase C generates diacylglycerol in addition to IP $_3$. Diacylglycerol also stimulates secretion, but this effect is mediated by stimulation of the recently identified enzyme protein kinase C rather than release of cytoplasmic Ca^{2+} (14, 33, 35). The hypothesis that the CDE diet results in reduced phospholipase C activity would, therefore, also suggest that the CDE diet causes a decrease in agonist-induced diacylglycerol generation. Support for this conclusion comes from our observation that acini prepared from mice fed the CDE diet can be stimulated to secrete amylase by addition of the synthetic diacylglycerol, OAG, and by the phorbol ester, TPA, equally well when compared to control acini.

In the pancreas, the Ca^{2+} -dependent and the protein kinase C-dependent pathways for stimulation of secretion are believed to complement each other with synergism occurring when both

pathways are stimulated, as for example by addition of a Ca^{2+} ionophore and one of the protein kinase C-stimulating phorbol esters (36–39). We have observed that both the IP $_3$ levels and the cytosolic Ca^{2+} levels can be modestly elevated by carbachol in CDE-treated acini, but even under these conditions amylase secretion remains below that noted after comparable elevations of cytosolic Ca^{2+} in acini prepared from mice fed the control diet (Fig. 2). This observation is consistent with the hypothesis that the CDE diet interferes with both IP $_3$ and diacylglycerol production by phospholipase C, in that the effect of decreased secretion cannot be completely attributed to a reduction in the degree of Ca^{2+} mobilization. This also may suggest the presence of a second lesion at a point in stimulus-secretion coupling beyond the release of Ca^{2+} in the cells. This observation is made more interesting by the fact that comparable secretion by control and CDE-treated acini can be elicited by activation of either the Ca^{2+} -dependent (ionophore) or protein kinase C (TPA or OAG) pathways, separately. This suggests that the disparity in secretion may result from a lack of synergism or interaction between the two pathways.

To determine the nature of the precise biochemical defect by which the CDE diet prevents IP $_3$ generation, several possible mechanisms must be examined. One possibility that we examined was that the CDE-induced lesion could cause a decrease in the amount of PIP $_2$ in the acinar cells, thereby depleting the substrate for phospholipase C. We found, however, that the level of PIP $_2$ (expressed as a percentage of total pancreatic phospholipid) was comparable between the control and CDE-treated mice. These determinations were based on measuring the mass of the phospholipids by assaying lipid phosphorus. Further, loading studies with [^3H]myoinositol in acini from control and CDE-diet mice revealed that the incorporation of the [^3H]myoinositol into cellular lipid was similar and, therefore, that PI-kinase and PIP-kinase activity was comparable between the two groups. These data demonstrate that the decrease in agonist-stimulated inositol phosphate generation in CDE-treated pancreatic acini is not due to a depletion of the substrate for phospholipase C, i.e., PIP $_2$.

Among other possibilities to be examined are alterations in the membrane phospholipid composition that could modulate phospholipase C activity. Membrane phospholipids have been reported to regulate phospholipase C activity *in vitro* (40, 41). *S*-adenosylethionine, a product formed after the CDE treatment is an ethyl-group donor and interferes with *S*-adenosylmethionine-dependent methylations (42). Such methylations are involved in choline synthesis. Thus, the CDE diet could lead to changes in membrane lipid composition and fluidity and thereby modulate phospholipase C activity. An alternative hypothesis involves the recently proposed involvement of a GTP-binding protein as a coupling step between receptor and phospholipase C (43–45). The CDE diet may alter the structure and/or function of this component leading to a loss in receptor-phospholipase coupling and, thus, diminished PI hydrolysis.

In summary, the observations reported in this communication demonstrate that the CDE diet interferes with stimulus-secretion coupling in mouse pancreatic acinar cells by preventing IP $_3$ (and possibly diacylglycerol) generation from PIP $_2$ by the action of phospholipase C. In addition, the decreased production of IP $_2$, IP $_1$, and diacylglycerols from the breakdown of PIP and PI could also have a role in explaining the effects of the CDE diet on secretion apart from a direct effect on cytosolic Ca^{2+} . To our knowledge, this is the first report of cellular pathology which

2. Irvine et al. (34) have reported that two isomers of IP $_3$ are produced after stimulation, the Ca^{2+} -releasing 1,4,5-IP $_3$ and a nonactive 1,3,4-IP $_3$. The column separation technique can not resolve these two compounds; therefore the IP $_3$ data include the measurement of both isomers.

can be related to a derangement in inositol phosphate generation. Inhibition of digestive enzyme secretion causes zymogen granules to accumulate eventually leading to fusion of these granules with lysosomes by crinophagy, intrapancreatic digestive enzyme activation, and acute hemorrhagic pancreatic necrosis (21, 22, 46, 47).

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