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Effect of Cholecystokinin-pancreozymin on the Nitric Oxide Synthase Activity and Cyclic GMP Level in Rat Pancreatic Tissue

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In pancreatic cells, NO formation is associated with increased levels of cGMP and endocrine/exocrine secretion. In the present study, the role of NO in the regulation of exocrine secretion was investigated in rat pancreatic tissues. Treatment of rat pancreatic tissue with cholecystokinin-pancreozymin (CCK-PZ) resulted in an significant increase in arginine conversion to citrulline, the amount of nitrite/nitrate, the release of amylase, and the level of cGMP. Furthermore, CCK-PZ-stimulated increase of amylase release and conversion of arginine to citrulline transformation were counteracted by the inhibitor of NO synthase, N^G-nitro-L-arginine methyl ester. The results on the time course of CCK-PZ- induced citrulline formation and rise of cGMP level indicate that NO synthase and guanylate cyclase are activated within the first seconds of stimulation. The kinetics of citrulline accumulation correlate well with those of cGMP rise, which further confirms the conclusion that NO mediates the response to CCK-PZ by cGMP.

Key word : Nitric oxide, Cholecystokinin-pancreozymin, Cyclic GMP, Exocrine secretion

INTRODUCTION

The enzyme responsible for the synthesis of Nitric Oxide (NO) from L-arginine in mammalian tissues is known as NO Synthase (EC.1.14.13.39) (Nathan and Xie, 1994). The nitrogen of NO by NO synthase comes from one of the two chemically equivalent guanidino nitrogens of L-arginine, producing L-citrulline as a coproduct. The synthesis of NO requires not only L-arginine and NADPH as substrates but also other cofactors such as Ca^{2+}, protoporphyrin IX haem, FMN, FAD, and BH_4 as well as calmodulin (Knowles and Moncada, 1994). The classical NO-mediated functions are endothelium-dependent relaxation (Furchgott and Zawadzki, 1980), neurotransmission (Garthwaite *et al.*, 1988), and cell-mediated immune response (Nathan and Hibbs, 1991).

NO derived from neuroendocrine, endocrine, and epithelial cells is involved in paracrine and autocrine regulation of neurotransmitter, polypeptide, and ion secretion. In pancreatic β cells, NO formation is associated with increased levels of cGMP and a lower threshold for D-glucose to induce insulin release (Schmidt *et al.*, 1992). NO synthase attenuates this potentiation of glucose-induced insulin release. Conversely, interleukin-1β induced NO inhibits glucose-stimulated insulin secretion by rat islet (Corbett *et al.*, 1993). In addition, both arginine and methylarginine stimulate insulin-release in the perfused rat pancreas.

The exocrine pancreatic secretion involves a variety of neurohormonal factors and is mediated by multiple regulatory pathways in the acinar cells (Solomon, 1987). Agonists such as carbachol, cholecystokinin-pancreozymin (CCK-PZ), and bombesin cause changes in cellular Ca^{2+} level and digestive enzyme secretion. The activation of Ca^{2+} influx is required for regulating free cytosolic Ca^{2+} concentration, for refilling the internal Ca^{2+} stores, and for the secretory response in pancreatic acinar cells (Muallem, 1989; Gukovskaya, 1994). The mechanism and process by which depleted Ca^{2+} stores activate influx is mediated by cGMP (Pandol and Schoeffield-Payne, 1990). Although, the mechanism of agonist-stimulated guanylate cyclase activation in these cells remains unknown, it is currently thought that most of the effects of NO in signal transduction are mediated by activation of guanylate cyclase.

The purpose of this investigation is to determine if NO synthesis is initiated in pancreatic tissues by treatment with CCK-PZ. Also it was to determine if NO functions as a signaling or effector molecule mediating exocrine pancreatic secretion.

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MATERIALS AND METHODS

Materials

[2,3,4,5-^{3}H]-L-arginine (57 mCi/mmole) and cyclic GMP assay kit were purchased from Amersham Life Science, U.S.A. Calmodulin, NADPH, cholecystokinin-pancreozymin (from porcine synthetic), Dowex-50W, (H^+ form, 200~400 mesh), PMSF, leupeptin, pepstatin A, sodium nitrite, sulfanilamide, N-(1-naphthyl) ehtylenediamine, tris[hydroxymethyl]aminomethane, and sodium acetate were obtained from Sigma Chemical Co., U.S.A. N^G-nitro-L-arginine methyl ester was from Calbiochem Co., U.S.A. All other chemicals were from commercially available sources.

Animals

Male Sprague-Dawley rats (200~250 g) were purchased from Ansung Cheil Inc. (Ansung, Korea) and housed under conditions of controlled temperature (22~24°C) and illumination (12-h light cycle starting at 8 A.M.) for 1 week in GLP room in college of pharmacy, Sungkyunkwan university.

Preparation and time of incubation of rat pancreatic tissue with CCK-PZ

Rats were killed by decapitation at the beginning of the experiments. Rat pancreatic tissues were quickly removed, cleaned and immersed in ice-cold Kreb's Ringer Bicarbonate (KRB) buffer, pH 7.4. Isolated pancreatic tissues in KRB buffer that was gassed with 5% CO_2 and 95% O_2 in 4 ml were incubated for indicated times at 37°C in absence or presence of CCK-PZ. After incubation was over, incubation media were collected to measure amylase activity released and the amount of nitrite. Incubated pancreatic tissues were frozen at -70°C and used for measuring nitric oxide (NO) synthase activities and cGMP levels.

Effect of the concentration of CCK-PZ on exocrine secretion in rat pancreatic tissue

Rat pancreatic tissues were isolated and incubated for 30 min in absence or presence of CCK-PZ (0.42×10^{-3}, 0.84×10^{-3}, 1.68×10^{-3}, 3.36×10^{-3}, 6.72×10^{-3}, 13.44×10^{-3} unit/0.5 g wet tissue) at 37°C as described. The medium was analyzed for amylase activity released and the amount of nitrite/nitrate. Rat pancreatic tissue was used for the assay of NO synthase.

Nitric oxide (NO) synthase assay (citrulline formation)

Activity of NO synthase was determined using an assay based on the conversion of ^{3}H-L-arginine to ^{3}H-L-citrulline as described (Bredt and Snyder, 1990). Rat pancreatic tissues were homogenized by glass homogenizer with Teflon pestle at 4°C in 4 volume (w/v) of ice-cold 50 mM Tris-HCl, pH 7.4, containing 0.1 mM EDTA, 0.1 mM EGTA, 0.1% 2-mercaptoethanol, 1 mM PMSF, 2 μM leupeptin and 1 μM pepstatin A. The homogenate was treated with Dowex-50 W Na^+ form (50X8-400, 200~400 mesh and prepared from the H^+ form), that had been pre-equilibrated with 50 mM Tris-HCl, pH 7.4 and centrifuged at 15,000×g for 20 min at 4°C. The supernatant was collected and used for NO synthase assay. Enzymatic reactions were carried out at 37°C in 50 mM Tris-HCl, pH 7.4, containing 50 μM L-arginine (approximately 200,000 dpm of L-[2,3,4,5-^{3}H]-L-arginine HCl : 57 mCi/mmole : Amersham Life Science), 1 mM NADPH, 1 μg calmodulin, and 0.6~1.0 mg supernatant protein in a final incubation volume of 100 μl. Reactions were initiated at 37°C by the addition of the supernatant. Reactions were terminated by the addition of 1 ml of ice-cold Dowex-50 W, Na^+ form (prepared from the H^+ form) which had been pre-equilibrated with 20 mM sodium acetate buffer, pH 5.5, containing 1mM citrulline, 2 mM EDTA and 0.2 mM EGTA (stop buffer), and the reaction mixtures were left for 5 min and centrifuged at 11,000×g for 10 min. The supernatant (300 μl) was collected into a suitable water-miscible scintillant and the radioactivity was counted in a Pharmacia 1209 Rackbeta liquid scintillation counter.

Nitrite determination

The amounts of nitrite/nitrate in the incubation medium were determined by using Griess reagent (0.5% naphthylethylenediamine dihydrochloride, 5% sulfanilamide, 25% H_3PO_4). Reactions were initiated by adding 1 ml of Griess reagent to samples (500 μl) at 22°C for 15 min. The absorbance at 540 nm was measured, using $NaNO_2$ solution as a standard.

Amylase assay

Assay for the enzymatic activity was carried out by the Bernfeld method (Bernfeld, 1955).

Determination of cGMP

The assay was based on the competition between unlabelled cGMP and a fixed quantity of the ^{3}H labelled compound for binding to an antiserum which has a high specificity and affinity for cGMP. The amount of labelled cGMP bound to the antiserum was inversely related to the amount of cGMP present in the assay sample, using commercially prepared kit from Amersham international plc.

Preparation of cGMP sample : Rat pancreatic tis-

sues were homogenized in 9 volumes (w/v) of ice-cold Hank's balanced salt solution (without calcium and magnesium) containing 139 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4 \cdot 7H_2O$, 0.44 mM KH_2PO_4, 0.37 mM $Na_2HPO_4 \cdot H_2O$, 4.16 mM $NaHCO_3$, 5.56 mM glucose, 0.05 mM phenol red and 5 mM EDTA, using glass homogenizer with Teflon pestle at 4°C. The homogenate was centrifuged at 1,000×g for 10 min at 4°C. The supernatant was diluted the ratio 1:10 with Hank's balanced salt solution and 1 ml was applied to SAX column which had been rinsed with 2 ml methanol and 2 ml distilled water. The column was washed with 3 ml methanol to remove interferring materials and eluted with 3 ml acidified methanol. The eluate was dried under nitrogen at 37°C and reconstituted in 0.5 ml of 50 mM Tris-HCl, pH 7.5, containing 4 mM EDTA.

Determination of cGMP : The reaction was carried out in 50 mM Tris-HCl, pH 7.5, containing 8 μM [8-^{3}H]guanosine 3',5'-cyclic phosphate (1.6 μCi : Amersham), 0.07~0.15 mg supernatant protein and antiserum (specific for cGMP) in a final incubation volume of 200 μl at 2~8°C for 30 min. Separation of the antibody-bound cGMP from the unbound nucleotide was achieved by 1 ml of a 60% saturated $(NH_4)_2SO_4$ solution, followed by centrifugation at 12, 000 rpm for 2 min. The precipitate, which contained the antibody- bound complex, was dissolved in 1.1 ml of distilled water and it was left for 5 min. The suspension (1 ml) was added to a suitable water-miscible scintillant, and it was counted in a Pharmacia 1209 Rackbeta liquid scintillation counter. The concentration of unlabelled cGMP in the sample was determined from a linear standard curve.

Pretreatment of rat pancreatic tissue with NG-nitro-L- arginine methyl ester (L-NAME)

Isolated pancreatic tissues in KRB buffer were incubated with or without L-NAME (1 mM/0.5 g wet tissue) for 15 min (Bennet *et al.*, 1992). CCK-PZ (8.4×10^{-3} unit/0.5 g wet tissue) was added and the mixtures were incubated for indicated time periods (15, 30, 45 min).

Protein determination

Protein contents of the enzyme preparations were measured by the Bradford method (Bradford, 1976), using bovine serum albumin as a standard.

Statistical analysis

The results were expressed as mean plus or minus the standard error. Statistical analysis was performed by using a two-tailed Student's t test. A difference with a p value of < 0.05 was considered statistically significant.

RESULTS

Effect of concentration of CCK-PZ on exocrine secretion in rat pancreatic tissue

Rat pancreatic tissues were isolated and incubated for 30 min in absence or presence of CCK-PZ. The results showed that CCK-PZ at concentrations ranging from 0.42 to 13.44×10^{-3} unit/0.5 g wet tissue significantly increased amylase release and the amount of nitrite (Table 1).

Effect of CCK-PZ on exocrine secretion in rat pancreatic tissue in relation to the incubation time

Effect of time on the NO synthase activity in rat pancreatic tissue : In CCK-PZ-treated rat pancreatic tissues, CCK-PZ significantly increased NO synthase activity (2.91±0.20 vs. 3.21±0.16 and 2.59±0.23 vs. 3.04±0.23 pmol/mg protein/min at 0.5 and 1 min incubation, respectively $p<0.05$) (Fig. 1).

Effect of time on the amounts of nitrite and the release of amylase into the incubation medium : In CCK-PZ-treated rat pancreatic tissues, the amount of nitrite was significantly increased as the incubation time increased (5, 10, 30, 60 min) (Fig. 2).

In CCK-PZ-treated rat pancreatic tissues, amylase release was significantly increased after 30 min of incubation (Fig. 3).

Effect of time on the cGMP level : In CCK-PZ-treated rat pancreatic tissues, cGMP level rose up to 2 times at the peak within 0.5 to 1 min after the addition of CCK-PZ, and it returned to the basal level at 5 min (Fig. 4).

Effect of L-NAME on exocrine secretion

Inhibitory effect of L-NAME on NO synthase activity in rat pancreatic tissue : Incubation of rat pan-

Table 1. Effect of concentration of CCK-PZ on amylase release, NO synthase activity and amount of nitrite in rat pancreatic tissue

CCK-PZ [$\times10^{-3}$ unit/0.5 g wet tissue]	Amylase release (μmol/ml/min)	Amount of nitrite (μM/15min)
-	7.02±0.32	8.21±1.09
0.42	8.84±0.44*	8.93±0.91
0.84	9.47±0.53*	10.79±0.14
1.68	10.01±0.48*	10.52±0.67
3.36	10.50±0.16*	11.47±0.55
6.72	10.84±0.21*	11.75±1.09
13.44	11.24±0.16*	15.41±0.68*

Rat pancreatic tissues were isolated and incubated for 30 min. The media were analyzed for amylase released as described in Bernfeld. Nitric oxide synthase activities were determined as described in Method. Results represent the mean±S.E. of duplicate determinations from 2 separate experiments.

*: $p<0.05$ vs. control (two-tailed Student's t-test)

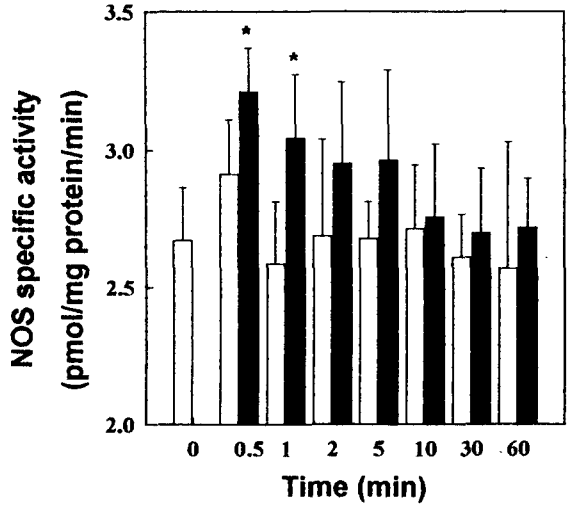

Fig. 1. Effect of time on the NO synthase activity in rat pancreatic tissue stimulated by CCK-PZ. The concentration of CCK-PZ in the incubation medium was 8.4×10^{-3} unit/0.5 g wet tissue. Results given are mean±S.E. of duplicate incubations and representative of 6-9 experiments. *: $p<0.05$ vs. control (two tailed Student's t-test), □: Control, ■: CCK-PZ at 8.4×10^{-3} unit/0.5 g wet tissue

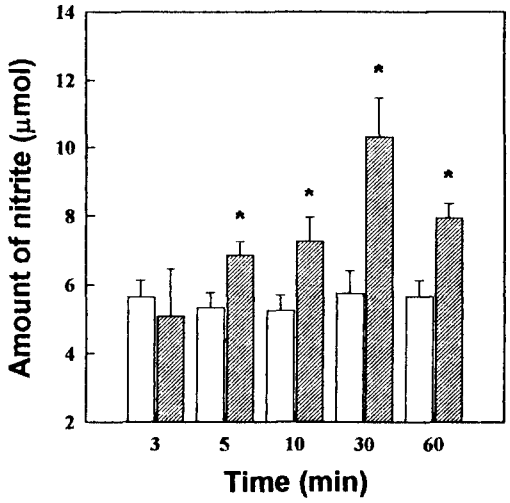

Fig. 2. Effect of time on the amounts of nitrite in the incubation medium of rat pancreatic tissue stimulated by CCK-PZ. Rat pancreatic tissues were preincubated in KRB buffer solution for 3 min, and then incubated with or without CCK-PZ for the indicated times. Results represent mean±S.E. of 7 separate experiments. *: $p<0.05$ vs. control (two-tailed Student's t-test), □: Control, ▨: CCK-PZ at 8.4×10^{-3} unit/0.5 g wet tissue

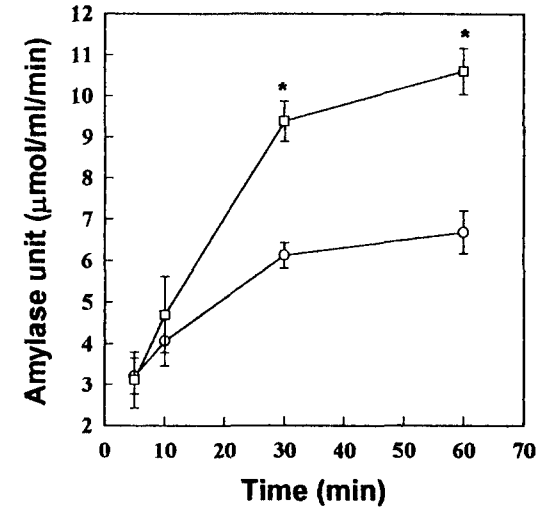

Fig. 3. Effect of time on the release of amylase into the incubation medium of rat pancreatic tissue stimulated by CCK-PZ. Rat pancreatic tissues were preincubated in KRB buffer solution for 3 min, and then incubated with or without CCK-PZ for the indicated times. Results represent mean±S.E. of 7 separate experiments. *: $p<0.01$ vs. control (two-tailed Student's t-test), O: Control, □: CCK-PZ at 8.4×10^{-3} unit/0.5 g wet tissue

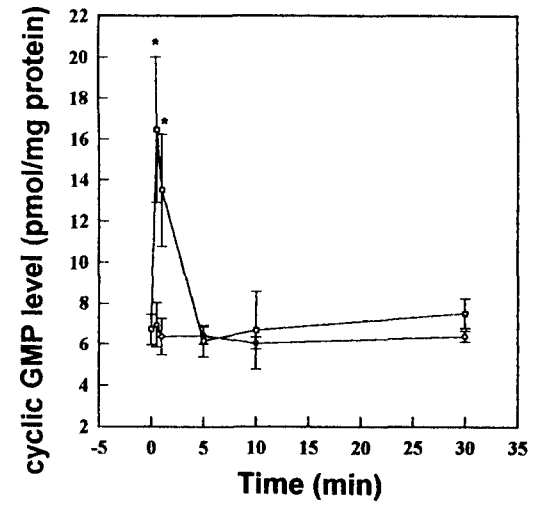

Fig. 4. Effect of time on the cGMP level in the rat pancreatic tissue incubated with CCK-PZ. Rat pancreatic tissues were preincubated in KRB buffer solution for 3 min, and then incubated with or without CCK-PZ for the indicated times. Results represent mean±S.E. of 6 separate experiments. *: $p<0.05$ vs. control (two-tailed Student's t-test), O: Control, □: CCK-PZ at 8.4×10^{-3} unit/0.5 g wet tissue

creatic tissues with an arginine derivative inhibitor of NO synthase, L-NAME (1 mM/0.5 g wet tissue), was found to counteract the increase of NO synthase activity caused by CCK-PZ (Fig. 5).

Inhibitory effect of L-NAME on the amylase release into the incubation medium : Pretreatment of rat pancreatic tissues with L-NAME reduced the CCK-PZ-stimulated amylase release (Fig. 6).

DISCUSSION

The observation in 1980 that rabbit aortal rings containing lining showed a significant and dose-dependent dilation in response to acetylcholine signals the new era of NO story. It was proposed at that time that relaxation of the isolated preparation of aorta and other blood vessels by acetylcholine requires the pres-

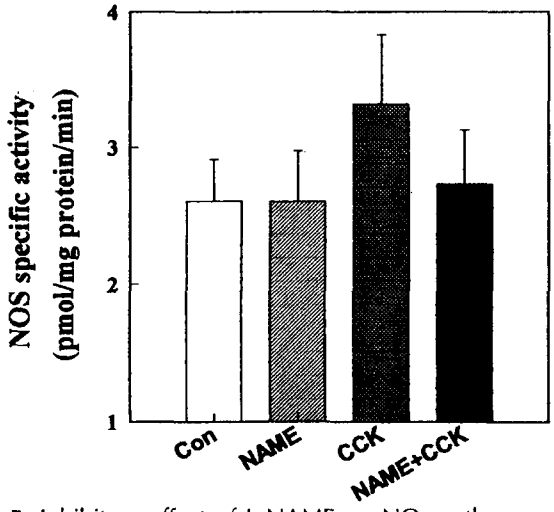

Fig. 5. Inhibitory effect of L-NAME on NO synthase activity in rat pancreatic tissue in incubated with CCK-PZ. Rat pancreatic tissues were preincubated in KRB buffer solution for 3 min, and then incubated with or without L-NAME (1 mM/0.5 g wet tissue) for 15 min. Subsequently, CCK-PZ (8.4×10^{-3} unit/0.5 g wet tissue) was added. NO synthase activity was measured after 60 min. Results given are mean±S.E. of duplicate incubations and representative of 7 experiments. □: Control, ▨: NAME 1 mM, ▧: CCK-PZ (8.4×10^{-3} unit/0.5 g wet tissue), ■: NAME 1 mM+CCK-PZ (8.4×10^{-3} unit/0.5 g wet tissue)

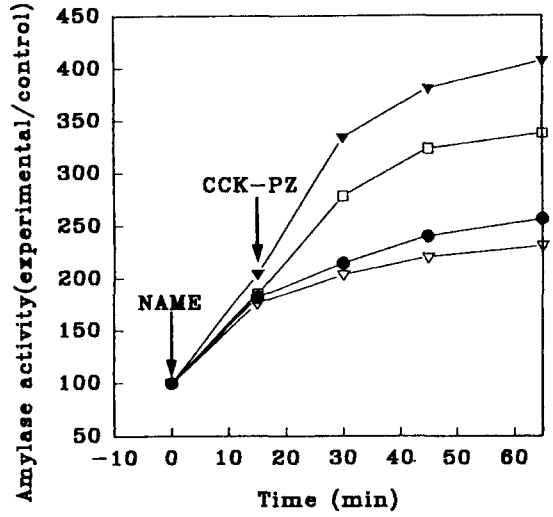

Fig. 6. Inhibitory effect of L-NAME on the amylase release into the incubation medium of rat pancreatic tissue incubated with CCK-PZ. Rat pancreatic tissues were preincubated in KRB buffer solution for 3 min, incubated with or without L-NAME (1 mM/0.5 g wet tissue) for 15 min, and then CCK-PZ (8.4×10^{-3} unit/0.5 g wet tissue) was added. Results given are means of values from 6-7 separate experiments with S.E. < 10%. ●: Control, ▽: NAME, ▼: CCK-PZ, □: NAME+CCK-PZ

ence of endothelial cells and that acetylcholine releases a highly lipophilic substance from these cells which diffuses to the adjacent smooth muscle cells to cause their relaxation. This substance was named endothelium-derived relaxing factor (EDRF), and was suggested to stimulates the formation of cGMP in the target cells via activation of cytosolic guanylate cyclase. Subsequently, the proof that the effect of EDRF released by endothelial cells and NO are identical was provided in 1987 by two groups of independent investigators. (Palmer *et al.*, 1987; Ignarro *et al.*, 1987).

During the last several years, a number of discoveries from many different research laboratories have proved that the major biological role of NO to be a neurotransmitter in the nervous system and other parts of the body (Moncada *et al.*, 1991), a potent vasodilating and cytoprotective substance, a mediator of endotoxin-induced cytotoxicity, and a substance involved in various disorders.

In addition, NO plays an important role in paracrine and autocrine regulation of neurotransmitter, protein, and ion secretion. NO can lead to an increase or decrease in the secretory response. Inhibition of secretion may follow the same mechanisms that inhibit increases of free intracellular Ca^{2+} in platelets. To stimulate secretion, NO may function by lowering the threshold concentration for an essential stimulant in the same way as it acts in long term potentiation.

For over 15 years, increased cGMP has been associated with stimulation of pancreatic enzyme secretion in pancreatic cells, however its direct role as a mediator in this process has remained to be controversial. The majority of effects of NO under physiological conditions appear to be mediated primarily by the activation of the intracelluar NO receptor guanylate cyclase (Garbers, 1992) resulting in the increase of cGMP. The results reported in this work provide a strong evidence to indicate that NO produced by CCK-PZ mediates the cGMP formation in rat pancreatic tissues. This conclusion is derived from the following findings : 1. CCK-PZ induced an significant increase in arginine conversion to citrulline (Fig. 1), the amount of nitrite (Fig. 2), amylase release (Fig. 3), and cGMP level (Fig. 4). 2. CCK-PZ-stimulated amylase release and the conversion of arginine to citrulline were antagonized by the inhibitor of NO synthase, L-NAME (Fig. 5, 6). The data on the time course of CCK-PZ- induced citrulline formation (Fig. 2) and the rise of cGMP indicate that NO synthase and guanylate cyclase are activated within the first seconds of stimulation. These results are in good agreement with the report by Gukovskaya and Pandol in demonstrating that NO production and the rise of cGMP are required for the Ca^{2+} influx by carbachol in pancreatic acinar cells. Therefore, activation of NO synthase is one of the early events in receptor-mediated cascade of reactions in pancreatic tissues and the kinetics of citrulline accumulation correlates well with those of cGMP rise, which further confirms the conclusion that NO mediates the

response to CCK-PZ by cGMP.

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