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NO/cGMP Pathway is Involved in Exocrine Secretion from Rat Pancreatic Acinar Cells

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The enzyme responsible for the synthesis of nitric oxide (NO) from L-arginine in mammalian tissues is known as nitric oxide synthase (NOS) (EC.1.14.13.39). In the present study, the role of NO in the regulation of exocrine secretion was investigated in rat pancreatic acinar cells. Treatment of rat pancreatic acinar cells with cholecystokinin-octapeptide (CCK-OP) resulted in an increase in the arginine conversion to citrulline, the amount of NO_x, the release of amylase, and the level of cGMP. Especially, CCK-OP-stimulated increase of arginine to citrulline transformation, the amount of NO_x and cGMP level were completely counteracted by the inhibitor of NOS, N^G-monomethyl-L-arginine (MMA), by contrast, that of amylase release was partially reduced. Furthermore, MMA-induced decrease of NOS activity and amylase release showed dose-dependent pattern. The data on the time course of CCK-OP-induced citrulline formation and cGMP rise indicate that NOS and guanylate cyclase were activated by treatment of CCK-OP. However, the mechanism of agonist-stimulated guanylate cyclase activation in acinar cells remains unknown. Therefore, activation of NOS is one of the early events in receptor-mediated cascade of reactions in pancreatic acinar cells and NO, not completely, but partially mediate pancreatic enzyme exocrine secretion.

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

INTRODUCTION

Nitric oxide (NO) plays an important role in a wide range of biological functions, including vasodilation, regulation of normal vascular tone, inhibition of platelet aggregation, neuronal transmission, cytostasis, hypotension associated with endotoxic shock, inflammatory response-induced tissue injury, mutagenesis, and formation of carcinogenic N-nitrosamines in mammals (Furchgott and Zawadzki, 1980 and Nathan and Hibbs, 1991). Recently, in pancreatic acinar cells, it is controversial if NO functions as a signaling molecule mediating pancreatic exocrine secretion. 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 (CCK), and bombesin cause changes in cellular Ca^{2+} and digestive enzyme secretion. Agonist-induced Ca^{2+} influx occurs as a result of the agonist ability to release Ca^{2+} from inositol-1,4,5-triphosphate-sensitive stores. 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). The mechanism and process by which depleted Ca^{2+} stores activate influx are 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 NO effects in signal transduction are mediated by activation of guanylate cyclase. Recently, Pandol and Shoeffield-Payne (1990) suggested that cGMP is sufficient to activate Ca^{2+} entry and may mediate the effect of agonists on Ca^{2+} entry in pancreatic acinar cells. Although these results suggest that Ca^{2+} store depletion or cGMP can regulate Ca^{2+} entry, the relationships between Ca^{2+} release from the store and the observed increase in cGMP are not known. Gukovskaya and Pandol (1993) have developed preliminary evidence that NO may play a role in mediating the increase in cGMP caused by the agonist under some conditions. Wrenn *et al.* (1994) also reported that NOS activity was detected in the extract of pancreatic acinar cells. In addition, our recent reports

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provided evidence that NO might be a putative mediator in pancreatic exocrine secretion (Seo *et al.*, 1995). In contrast, Hitoshi *et al.* (1997) demonstrated that NO/cGMP pathway is uncoupled on carbachol and CCK-stimulated Ca^{2+} entry and amylase secretion and that the NOS system is either not present or not functioning in rat pancreatic acinar cells. Therefore, the role of NO in exocrine secretion from rat pancreatic acinar cells has been unclear at present. In this study, we tried to establish if NO functions as a signaling molecule mediating pancreatic exocrine secretion by determining the change of amylase secretion, NOS activity and cGMP level upon stimulating pancreatic acinar cells with CCK-OP, one of the strong secretagogues.

MATERIALS AND METHODS

Materials

[2,3,4,5-^{3}H]-L-arginine (57 Ci/mmole) and cyclic GMP assay kit were purchased from Amersham Life Science, U.S.A. Collagenase (Type V), α-chymotrypsin (Type I-S), hyaluronidase (Type I-S), trypsin inhibitor (Type II-S, Soybean), N^G-monomethyl-L-arginine, cholecystokinin-octapeptide, Dowex-50W, (H^+ form, 200~400 mesh), and other reagents were purchased from Sigma Chemical Co., USA.

Experimental animals and cell isolation

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. To measure NOS activity, cGMP level and amylase activity, acinar cells from rat pancreas were incubated and divided into medium and pellet. Rats were fasted overnight (14~18 h) prior to use, decapitated and exanguinated prior to removal of the pancreas. The incubation medium used for isolation was a modified Krebs-Henseleit bicarbonate (KHB) medium containing 118 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 14 mM glucose, 0.01% (w/v) soybean trypsin inhibitor and minimal Eagles medium (MEM) amino acid supplement. Dissociation medium also contained 0.22 mg/ml of purified collagenase, 0.1 mg/ml chymotrypsin, 1.8 mg/ml hyaluronidase and 0.1 mg/ml trypsin inhibitor in KHB medium. Dissociation medium (5 ml) was injected into the parenchyma of the pancreas with a 27-g needle to distend the tissue, and the pancreas was shaken in a 25 ml polycarbonate Erlenemeyer flask for 10 min (120 cycles/min) at 37°C. Excess medium was withdrawn and 5 ml fresh dissociation medium added, followed by incubation with shaking for another 35~40 min. Acinar cells were then dissociated by sucking up and down through polypropylene pipette and filtered through a 150-μm mesh nylon cloth using an extra 10 ml of medium without enzymes but with 1% bovine serum albumin (BSA) as a rinse. Acinar cells were purified by centrifugation (5 min at 50 g) through KHB containing 4% BSA followed by washing with the same medium. Thereafter, the acinar cells were suspended in HEPES bufferd Ringer (HR) similar to KHB but without bicarbonate and containing 10 mM HEPES, pH 7.35, which was equilibrated with 95% O_2 (Williams *et al.*, 1978).

Amylase assay

Dispersed acinar cells from one pancreas were suspended in 50~100 ml of incubation solution. At appropriate times 500 μl of cell suspension was centrifuged at 10,000×g for 5 s in a microcentrifuge. Supernatant (100 μl) was added to 900 μl of diluent solution (phosphate buffer, pH 6.8 containing 1.5% NaCl). Reactions were initiated at 30°C by addition of phosphate buffer, pH 6.8, containing 1% starch solution in a final incubation volume of 300 μl. Reactions were terminated by the addition of 300 μl of 1% dinitrosalicylic acid (DNSA) solution, containing 0.4 M NaOH and 1 M potassium sodium tartrate, and then mixtures were boiled at 100°C for 5 min and cooled in ice-bath. The absorbance at 540 nm was measured, using maltose as a standard (Bernfeld, 1955). Amylase release was expressed as the percentage of total amylase activity according to the following formula: ((Experimental release-Spontaneous release)/(Maximal release-Spontaneous release))×100, here, maximal release represents total amylase activity after sonication in ice bath.

NOS assay ([^{3}H]citrulline accumulation)

[^{3}H]citrulline accumulation was determined as described previously (Gukovskaya and Pandol, 1994). Cells isolated from one pancreas were suspended in 5 ml of the incubation solution and incubated with [^{3}H] arginine (3 μCi/ml) for 20 min. At zero time point and at the indicated times after addition of CCK-OP, 0.25 ml aliquots from each tube were removed and mixed with 0.9 ml ice-cold buffer containing 5 mM arginine and 4 mM EDTA. This mixtures were centrifuged at 10,000×g for 30 s. Supernatants were aspirated, and 1 ml of a 1 M trichloroacetic acid was added to the pellet. The samples were sonicated and centrifuged (10,000×g, 20 min). The supernatants were collected, and the trichloroacetic acid was extracted three times with 2 ml of diethyl ether. Aliquots (0.5 ml) of the extracts were neutralized with 2 ml of 20 mM sodium acetate buffer (pH 5.5), and the total

volume was applied to 1 ml Dowex 50W-X8 column preequilibrated with the same buffer. The quantity of [^{3}H]citrulline in the collected eluate was determined by liquid scintillation counting.

NO analysis

NO produced by rat pancreatic acinar cells was measured by NO Analyzer (Model 7020, ANTEK Instruments, INC.). Briefly, isolated rat pancreatic acinar cells were incubated with indicated agents, sonicated and then centrifuged (10,000×g, 20 min). The 10 μl of the supernatant was injected directly into a hot vanadium (III) reduction solution which can reduce nitrite to NO. Nitrate in the supernatant is also rapidly reduced after heating to 80~90°C. The chemiluminescence of NO reduced from nitrogen oxides in supernatant mixture was measured (Cox, 1980).

Determination of cGMP

Dispersed acinar cells from the pancreas of one rat were suspended in 10~20 ml of incubation solution and incubated at 37°C with indicated agents. At indicated times, 0.25 ml aliquots were removed and centrifuged at 10,000×g for 10 s using microcentrifuge. The supernatant was aspirated and 0.5 ml of iced ethanol was added to the pellet. After centrifugation (12,000×g, 20 min), the cGMP was determined in 150 μl aliquots of the supernatant using Amersham radioimmunoassay kit. The reaction was carried out in 50 mM Tris-HCl, pH 7.5, containing 8 μM [8-^{3}H] cGMP (1.6 μCi: Amersham), sample source 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 60% saturated $(NH_4)_2SO_4$ solution, followed by centrifugation at 10,000×g 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. Values for each experimental sample were expressed as a ratio to the control value (experimental to control).

Pretreatment of rat pancreatic acinar cells with MMA

Isolated pancreatic acinar cells in incubation medium were preincubated with or without MMA (1 mM) for 15 min and then, CCK-OP (10^{-10} M) was added for indicated time periods (1, 3, 5, 10, 15 min).

Statistical analysis

The results were expressed as mean plus or minus the standard error (% of total for amylase, % of basal for NOS and experimental to control ratio for cGMP). 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

Isolation of rat pancreatic acinar cells

Rat pancreatic acinar cells were isolated by trypsin digestion as described in Method. Because of the three-dimensional structure of the acinar cells, it is impossible to accurately count cells. Trypan blue was almost totally excluded by acinar cells for indicated incubation time. The increase in amylase release from dispersed acinar cells by secretagogues were substantially more responsive than that from single acinar cells. During 30 min incubation at 37°C, amylase release with CCK-OP from dispersed acinar cells was approximately 50%, which was five-fold larger than that from single acinar cells (data not shown). To determine the optimal concentration of secretagogues (CCK-OP and carbachol), the amylase release from dispersed acinar cells was measured as described in Method. The maximal increase caused by CCK-OP (10^{-10} M) and carbachol (10^{-5} M) was 57.35% and 17.37%, respectively (Fig. 1).

Effect of MMA on exocrine secretion from rat pancreatic acinar cells stimulated with CCK-OP

Time course effect of MMA pretreatment on NOS

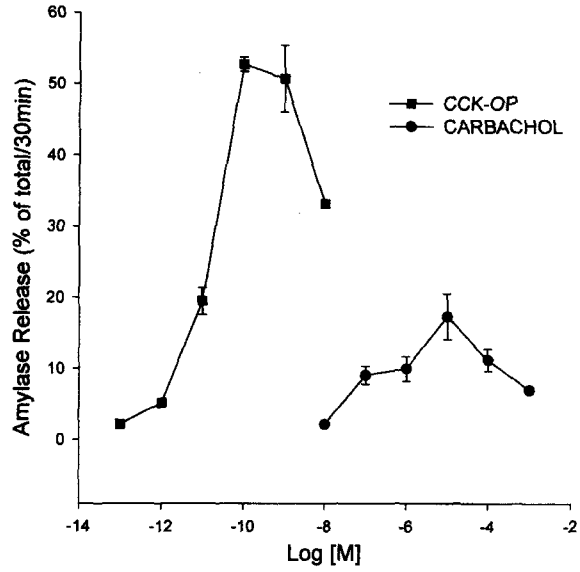

Fig. 1. Dose-response curves of secretagogue-stimulated amylase release in rat pancreatic acinar cells. Dispersed acinar cells were suspended in incubation solution and 1 ml aliquots were incubated with the indicated concentrations of secretagogues for 30 min at 37°C. The amount of amylase released into medium was measured as described in Method. Results represent mean±S.E. of 5 separate experiments.

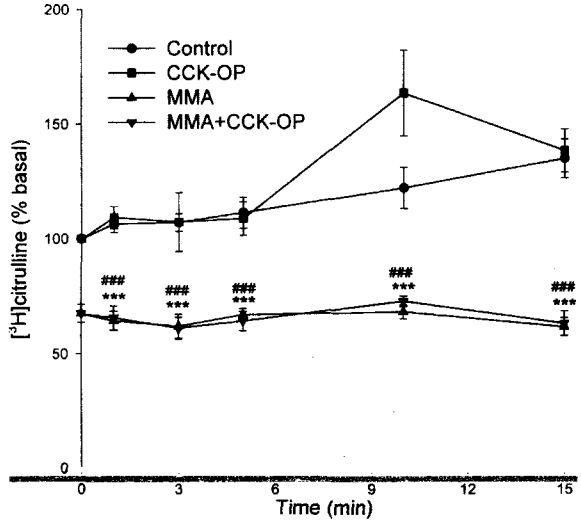

Fig. 2. Time course effect of MMA pretreatment on NOS acitvity. Acinar cells were preincubated in HEPES buffer solution for 15 min with or without 1 mM MMA and then stimulated with 10^{-10} M CCK-OP for indicated time. NOS assay was performed as described in Method. The value 100% corresponds to the zero-time or basal [^{3}H]citrullline levels. Results represent mean±S.E. of 10 separate experiments. ***p<0.001 vs. control, ###p<0.001 vs. CCK-OP (two-tailed Student's t-test)

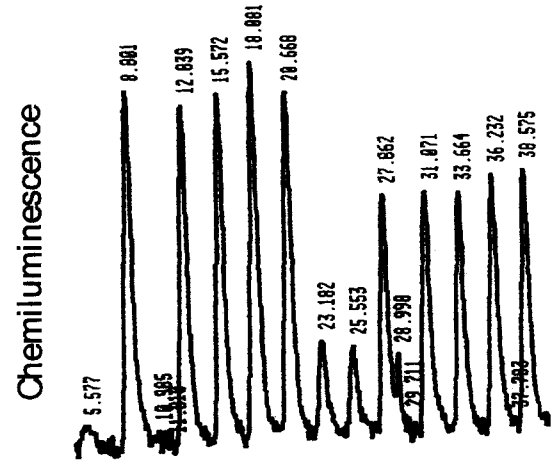

Fig. 3. Determination of NO formed by NOS in acinar cells by NO analyzer. Acinar cells were preincubated for 15 min with or without 1 mM MMA and further incubated in the presence or absence of 10^{-10} M CCK-OP. After incubation, acinar cells were sonicated and centrifuged to detect total amounts of nitrite/nitrate. The 10 μl of each supernatant was injected directly into the hot vanadium (III) reduction solution. The chemiluminescence of NO reduced from nitrite/nitrate in reaction mixture was measured. C: CCK-OP, M: MMA+CCK-OP, BL: Blank

activity: To determine whether the increase in amylase secretion by CCK-OP may be mediated by NO, NOS activity was measured using [^{3}H]citrulline accumulation method upon stimulating acinar cells with CCK-OP. CCK-OP activated NOS activity transiently with maximum at 10 min (122.2±9.0 vs. 163.7±18.7, % basal), which was significantly decreased to 70% of basal level by the inhibitor of NOS, MMA (1 mM) as the incubation time changed. Furthermore, MMA pretreatment either to untreated or CCK-OP treated cells decreased to 70% of basal level suggesting that NOS lie on CCK-OP-induced signaling pathway (Fig. 2).

Determination of NO formed by NOS in rat pancreatic acinar cells: To confirm the change of NOS activity, NO_x was determined using ANTEK NO analyzer (Model 7020). Consistent with the above data, the amount of NO was also increased by CCK-OP treatment, which was decreased by MMA (1 mM) as the incubation time changed (1, 3, 5, 10, 15 min) (Fig. 3).

Concentration-response dependence for MMA inhibition of NOS activity in rat pancreatic acinar cells: To determine dose-response of MMA, rat pancreatic acinar cells were pretreated for 15 min with increasing concentrations of MMA and then stimulated with CCK-OP for 1 min. NOS activity was significantly decreased concentration-dependently by MMA between 10^{-4} M and 10^{-3} M (72.12±7.80, 65.33±4.80, % basal) (Table I, Fig. 4).

Table I. Inhibitory effect of the concentration of MMA on amylase release, NOS activity and cGMP level in rat pancreatic acinar cells stimulated by CCK-OP

MMA [logM]	Amylase release (% of total, 30 min)	NOS ([^{3}H]citrulline, % basal, 1 min)	cGMP (experimental/control, 1 min)
-	53.60±7.59	100	1
-7	53.86±2.57	110.03±8.70	0.80±0.14
-6	53.62±1.19	100.23±5.00	0.96±0.02
-5	45.51±1.85*	89.02±5.50	0.95±0.17
-4	37.70±4.90*	72.12±7.80*	1.04±0.25
-3	35.49±1.01**	65.33±4.80**	0.71±0.14

The concentration of CCK-OP in the incubation medium was 10^{-10} M. Rat pancreatic acinar cells were isolated and incubated for indicated time. The media were analyzed for amylase released as described in Bernfeld. NOS activities and cGMP level were determined after sonication as described in Methods. Results represent the mean±S.E. from 3 separate experiments. *p<0.05, **p<0.01 vs. control (two-tailed Student's t-test)

Effect of time course on the cGMP levels in rat pancreatic acinar cells in the absence or presence of CCK-OP

Another candidate as a mediator of CCK-OP signal transduction pathway, cGMP was assayed when rat pancreatic acinar cells was stimulated with CCK-OP. cGMP level significantly rose up to 1.4 times at 1 min after the addition of CCK-OP (1.19±0.07 vs. 1.67±0.12, experimental/control) and then it returned to the basal level at 5 min. Futhermore, this rise was

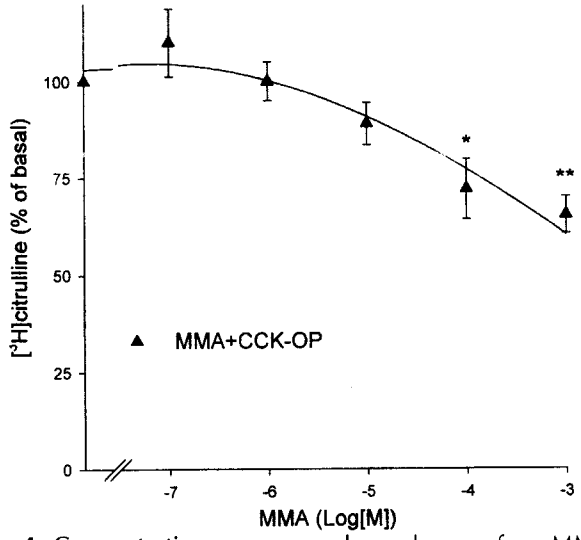

Fig. 4. Concentration-response dependence for MMA inhibition of NOS activity in rat pancreatic acinar cells. Acinar cells were preincubated in HEPES buffer solution for 15 min with various concentrations of 1 mM MMA. [^{3}H] citrulline was measured at 1 min after stimulation with 10^{-10} M CCK-OP as described in Method. Results represent mean ±S.E. of 3 separate experiments. *p<0.05, **p<0.01 vs. control (two-tailed Student's t-test)

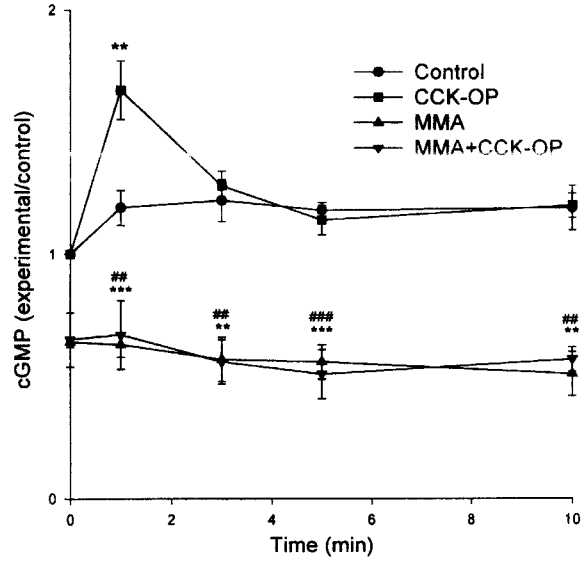

Fig. 5. Changes in cGMP level in response to CCK-OP in rat pancreatic acinar cells preincubated with or without MMA. Dispersed acinar cells were preincubated for 15 min with or without 1 mM MMA and then 10^{-10} M CCK-OP was added. The pellet was sonicated and analyzed for cGMP level as described in Method. Basal level for cGMP was 4 pmol/tube. Results represent mean±S.E. of 6 separate experiments. **p<0.01, ***p<0.001 vs. control, ##p<0.01, ###p<0.001 vs. CCK-OP (two-tailed Student's t-test)

significantly decreased by MMA (1 mM) as the incubation time changed. Furthermore, MMA pretreatment to untreated as well as CCK-OP treated cells decreased to 70% of basal level similarly with NOS activity. These results indicate that cGMP is a mediator of CCK-OP-stimulated signal transduction pathway (Fig. 5).

Effect of time course on the amylase release in rat pancreatic acinar cells

Time course effect of MMA on the amylase release into the incubation medium of rat pancreatic acinar cells stimulated by CCK-OP: To establish whether NO and cGMP are involved in CCK-OP-induced pancreatic exocrine secretion, the change of CCK-OP-stimulated amylase secretion was determined by pretreatment of rat pancreatic acinar cells with MMA. CCK-OP alone showed a significant amylase release for incubation time (3, 5, 10, 15, 30 min: 10.30±1.86 vs. 25.20± 1.97, 10.60±3.65 vs. 26.70±2.36, 14.30±1.30 vs. 37.90±1.93, 14.40±1.19 vs. 45.90±1.30, 17.10± 2.13 vs. 53.60±3.99, % of total), which was partially decreased by pretreatment with MMA (1 mM) as the incubation time changed. In contrast, NOS activity and the synthesis of cGMP were completely blocked under the same condition. In untreated-acinar cells, amylase release was also partially decreased by pretreatment of MMA (Fig. 6).

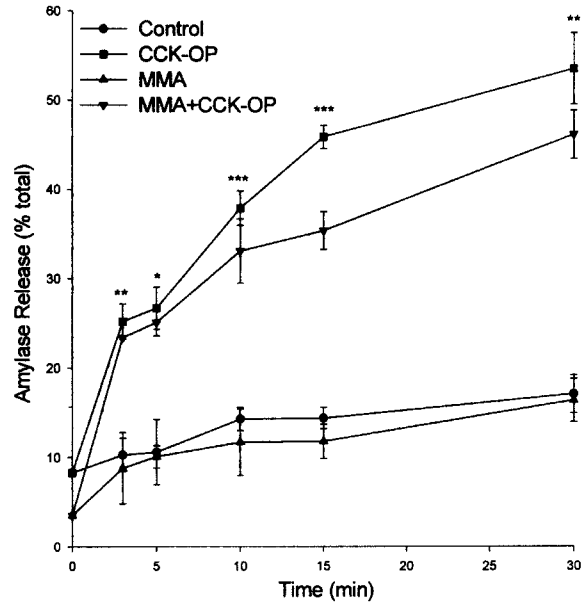

Fig. 6. Time course effect of MMA on the amylase release into the incubation medium of acinar cells stimulated by CCK-OP. Prepared acinar cells were preincubated for 15 min with or without 1 mM MMA and then 10^{-10} M CCK-OP was added. The medium was analyzed. Results represent mean±S.E. of 5 separate experiments. *P<0.05, ** P<0.01 ***P<0.001 vs. control (two-tailed Student's t-test)

Concentration-response dependence for MMA inhibition of amylase release in rat pancreatic acinar cells: In CCK-OP-treated rat pancreatic acinar cells, amylase release was significantly decreased concentration-dependently at 10^{-5} M, 10^{-4} M and 10^{-3} M (45.51±1.85, 37.70±4.90, 35.49±1.01, % of total)

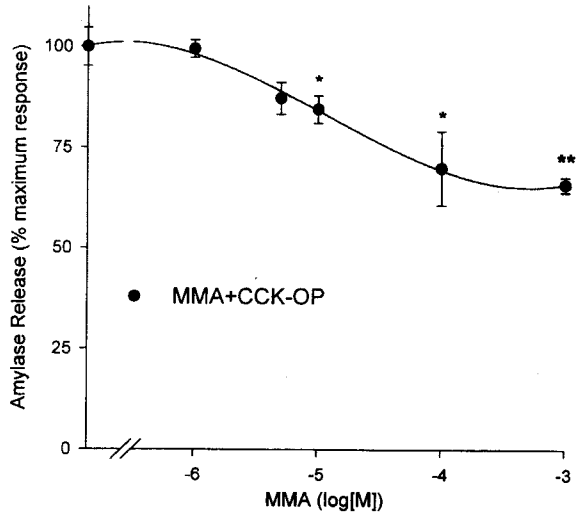

Fig. 7. Concentration-response dependence for MMA inhibition of amylase release in rat pancreatic acinar cells. Acinar cells were preincubated in HEPES buffer solution for 15 min with 1 mM MMA and amylase activity was measured at 5 min after stimulation with 10^{-10} M CCK-OP. Results represent mean±S.E. of 3 separate experiments. *p <0.05, **p<0.01 vs. control (two-tailed Student's t-test)

(Table I, Fig. 7). Together those results suggest that NO might partially mediate pancreatic amylase secretion and multiple pathways exist in pancreatic exocrine secretion.

DISCUSSION

During the last several years, a number of discoveries from many different researches have revealed that the major biological role of NO as 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. For example, inhibition of secretion may follow the same mechanisms that inhibit increases of free intracellular Ca^{2+} in platelets. To stimulate secretion, NO may function similarly as the way it acts in long term potentiation by lowering the threshold concentration for an essential stimulant.

For over 15 years, increase of cGMP has been associated with stimulation of pancreatic acinar cells, but its direct role as a mediator in this process has remained to be controversial. Pandol and Shoeffield-Payne (1990) reported that cGMP mediates the agonist-stimulated increase in plasma membrane calcium entry. In addition, Gukovskaya and Pandol (1994) suggested that NO production regulate cGMP formation and calcium influx in pancreatic acinar cells. Xin Xu *et al.* (1994) also reported that depletion of intracellular Ca^{2+} stores activates NOS to generate cGMP and regulate Ca^{2+} influx. These ideas indicated that NO/cGMP system is coupled with enzyme secretion in pancreatic acinar cells. However, Hitoshi *et al.* (1997) demonstrated that, in the rat pancreatic acinar cells, the NOS system is either not present or not functioning, that exogenous NO is capable of increasing endogenous cGMP by activating the soluble guanylate cyclase system, which results in modest Ca^{2+} transients and increases in amylase secretion, and that the NO/cGMP system is not linked to the signal transduction pathway activated by carbachol and CCK-OP. The reason why it is difficult to explain the connection between NO/cGMP system and enzyme secretion in pancreatic acinar cells is that the amount of NO produced by NOS in pancreatic acinar cells is very small and rapidly oxidized, diffused to other cells.

The results obtained in this work provide evidence that NO produced partially mediates the stimulation of CCK-OP by cGMP formation in rat pancreatic acinar cells. CCK-OP induced an increase in [^{3}H] arginine conversion to [^{3}H]citrulline (Fig. 2), cGMP level (Fig. 5) and amylase release (Fig. 6). In CCK-OP-stimulated pancreatic acinar cells, the amount of NO and cGMP levels were completely antagonized by the inhibitor of NOS, MMA (Fig. 2, 5), amylase release was partially inhibited by the same inhibitor (Fig. 6). CCK-OP-stimulated conversion of [^{3}H]arginine to [^{3}H] citrulline and amylase release were antagonized dose-dependently by the inhibitor of NOS, MMA (Table I, Fig. 4, 7). The data on the time course of CCK-OP-induced [^{3}H]citrulline formation (Fig. 2) and the rise of cGMP (Fig. 5) indicate that NOS and guanylate cyclase are activated within the seconds of stimulation. Therefore, activation of NOS is one of the early events in receptor-mediated cascade of reactions in pancreatic acinar cells. But, the kinetics of citrulline accumulation and cGMP rise further confirms the conclusion that NO mediates the response to CCK-OP by cGMP. Also, the fact that CCK-OP-induced amylase release was partially antagonized by MMA indicated that multiple pathways may regulate pancreatic exocrine secretion. Further studies should clarify the more precise role and mechanism of NO in the overall scheme of secretory regulation in the pancreatic acinar cells.

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