Arch. Pharm. Res. Vol.19, No.3, pp. 201-206, 1996

Vasoactive Intestinal Peptide (VIP)-induced Enzyme Secretion in Rat Pancreatic Tissue is not associated with Activation of Nitric Oxide Synthase (NOS) and Increase in Cyclic GMP Level

Tae Kyun Nam, Jeung whan Han, Suk Woo Nam, Dong-Wan Seo, Young Jin Lee, Young Kwon Ko and Hyang Woo Lee

College of pharmacy, Sungkyunkwan University, Suwon 440-746, Korea

(Recieved February 16, 1996)

Nitric oxide (NO) is thought to be a second messenger involved in secretion. Upon stimulating pancreatic acinar cells with cholecystokinin- pancreozymin (CCK-PZ), NO formation has been shown to be associated with increased levels of cGMP (Seo *et al.*, 1995). To elucidate the signaling pathway of VIP-induced enzyme secretion, we investigated the NO and cGMP synthesis steps as potential steps where two signal pathways triggered by CCK-PZ and VIP interact. The results obtained in this work provide evidence that increase in pancreatic enzyme secretion by treatment with VIP has no relationship with NOS activity and cGMP level. This conclusion was derived from the following findings that VIP treatment of rat pancreatic tissue increased amylase release as well as protein output in a dose- and time-dependent manner, whereas NOS activity and cGMP synthesis were not affected by VIP treatment as monitored by NOS activity assay and determining cGMP level, which was further confirmed by a NOS-inhibitor study. Consequently, CCK-PZ or VIP increases enzyme secretion in rat pancreatic tissue, but the two hormones are different in their mode of action. Together the results suggest that signaling pathway of VIP-induced enzyme secretion might either bypass the NO and cGMP synthesis steps or lie on a distinct pathway from CCK-PZ-induced pathway.

Key word : Nitric oxide, Vasoactive intestinal peptide, Cyclic GMP, Exocrine secretion

INTRODUCTION

NO is generated from the terminal guanidino nitrogen of L-arginine by an N^{ω}-hydroxy-L-arginine intermediate yielding citrulline. This reaction is catalyzed by nitric oxide synthase (EC 1.14.13.39) (Kiechle and Malinski, 1993). The classical NO-mediated functions are endothelium-dependent relaxation, neurotransmission, and cell-mediated immune response (Nathan and Xie, 1994). Most responses elicited by NO are due to the activation of soluble guanylyl cyclase leading to the accumulation of cGMP in the target cells (Schmidt and Walter, 1994). In contrast to the above, NO derived from neuroendocrine, endocrine, and epithelial cells is involved in paracrine and autocrine regulation of neurotransmitter, polypeptide, and ion secretion.

The role of NO in the pancreatic exocrine secretion has not been clearly determined. The control of pancreatic exocrine secretion is generally believed to be exerted chiefly by a variety of gastrointestinal hormones and vagal stimulation. Gastrointestinal hormones involved in control of pancreatic secretion include CCK-PZ, VIP, and secretin. CCK-PZ and muscarinic cholinergic agents stimulate enzyme secretion from the pancreas (Iwatsuki *et al.*, 1986). Since CCK-PZ increases cGMP levels prior to amylase release in pancreas (Christophe *et al.*, 1976) and since muscarinic cholinergic agents increase cGMP in several tissues including the pancreas (Haymovis and Scheele, 1976), cGMP rather than cAMP may be an intracellular mediator in the process of enzyme secretion from the pancreas. However, pancreatic secretion of HCO_3^- into the duodenum by secretin or VIP is mediated through cyclic AMP (Holst *et al.*, 1979).

On the other hand, recent evidence suggests that VIP also stimulates pancreatic enzyme secretion as CCK-PZ, but its mechanism remains unknown (Meyer-Alber *et al.*, 1994). VIP is found in nerves in the alimentary canal from the esophagus to the rectum, and in ancillary organs such as pancreas and gall bladder. The greatest number of VIP-immunoreactive

Correspondence to: Hyang Woo Lee, College of pharmacy, Sungkyunkwan University, Suwon 440-746, Korea

cells is found in the small intestine, particularly the jejunum and the ileum, and also in the colon. It is also found in some blood vessels and in the bronchi (Kruk and Pycock, 1991). It relaxes a variety of smooth muscles and antagonizes the effects of smooth muscle constrictor agents. In the pancreas, VIP is associated with both endocrine and exocrine secretion. As its endocrine function, it causes release of insulin, glucagon and somatostatin. On the other hand, VIP, which increases pancreatic fluid and bicarbonate secretion like secretin (Holst *et al.*, 1984), has been shown to stimulate enzyme secretion (Meyer-Alber *et al.*, 1994) in exocrine pancreatic secretion as CCK-PZ. Also secretin and VIP have been shown to potentiate the effect of secretagogues that increase intracellular calcium, such as acetylcholine and CCK-PZ. Thus, although these two groups of hormones act by distinct pathways, they interact at an unknown step, resulting in potentiation of their individual effects(Schulz and Stolz, 1980).

The purpose of this study is to further define the relationship between enzyme secretion and NOS signaling pathway in rat pancreatic tissues by treatment with VIP that stimulate enzyme secretion in pancreas.

MATERIALS AND METHODS

Materials

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

Methods

Preparation and incubation of rat pancreatic tissue in the absence or presence of VIP : Rats were sacrificed 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 the absence or presence of VIP. After incubation was over, incubation media were collected to measure amylase activity released and protein output. Incubated pancreatic tissues were frozen at -70°C and used for measuring NOS activities and cGMP levels.

Effect of the concentration of VIP on exocrine secretion in rat pancreatic tissue : Rat pancreatic tissues were isolated and incubated for 30 min in the absence or presence of VIP (0.5×10^{-7}, 1×10^{-7}, 2×10^{-7}, 4×10^{-7}, 8×10^{-7} M/0.5 g wet tissue) at 37°C as described. The media were analyzed for amylase activity released and protein output. Rat pancreatic tissue was used for the assay of NOS.

Pretreatment of rat pancreatic tissue with N^G-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). VIP (2×10^{-7} M/0.5 g wet tissue) was added and the mixtures were incubated for indicated time periods (15, 30, 45 min).

Enzymatic assay : Activity of NOS 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). Assay for the enzymatic activity was carried out by the Bernfeld method (Bernfeld, 1955). Protein contents of the enzyme preparations were measured by the Bradford method (Bradford, 1976), using bovine serum albumin as a standard. The assay of cGMP level 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 Life Science, U.S.A.

Data analysis : The data were expressed as mean ± 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

Rat pancreatic tissues were incubated with increasing concentrations of VIP for 30 min, and then the change of exocrine secretion in rat pancreatic tissue by VIP was determined. Amylase release and protein output for various concentrations of VIP were measured as a criterion of pancreatic enzyme secretion. Both amylase release and protein output were increased in a dose-dependent manner by VIP (Table I). The concentration of VIP, 2.0×10^{-7} M/0.5 g wet tissue was chosen for the following experiments as proper concentration of VIP, because it was the most effective concentration as compared with the others. After incubation of rat pancreatic tissue with VIP for various times, we tested for the ability of VIP to release pancreatic enzyme secretion. Amylase release significantly was increased from 30 min and 60 min of incubation (Fig. 1). Similarly, the protein output was also significantly elevated at all the in-

Table I. Effect of the concentration of VIP on amylase release and protein output in rat pancreatic tissue

VIP [$\times 10^{-7}$ M/0.5 g wet tissue]	Amylase release (μmol/ml/min)	Protein output (μg/0.5 g wet tissue)
-	7.66±036	538±27
0.5	8.23±0.43	567±56
1.0	8.19±0.37	581±18
2.0	8.60±0.23	645±28
4.0	8.45±0.43	661±42
8.0	8.26±0.31	722±49

Rat pancreatic tissues were isolated and incubated for 30 min. The media were analyzed for amylase released and measured for protein output as described in Materials and Methods. Results represent the mean±S.E. of duplicate determinations from 2 separate experiments.

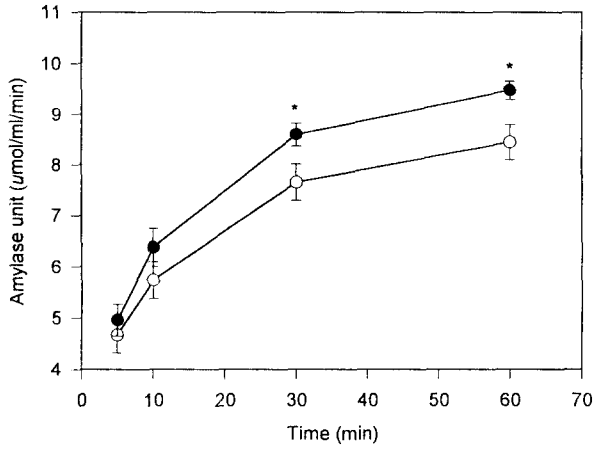

Fig. 1. Effect of time course on the amylase release into the incubation medium of rat pancreatic tissue in the absence or presence of VIP. Rat pancreatic tissues were preincubated in KRB buffer solution for 3 min, and then incubated with or without VIP for the indicated times. Amylase activity released into the incubation medium was measured as described in Materials and Methods. Results represent mean±S.E. of 7 separate experiments. *: $p<0.05$ vs. control (two-tailed Student's t-test)
○—○: Control, ●—●: VIP 2×10^{-7} M/0.5 g wet tissue

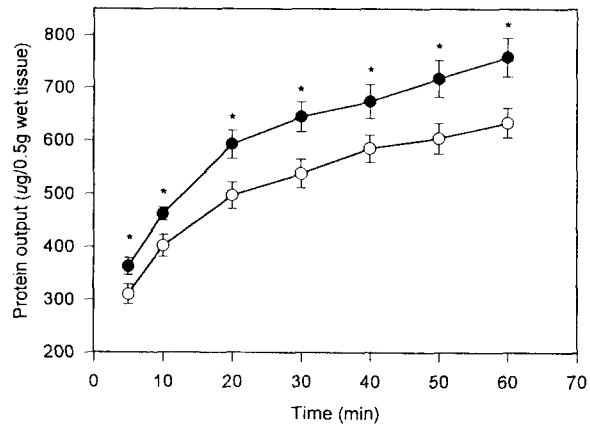

Fig. 2. Effect of time course on the protein output into the incubation medium of rat pancreatic tissue in the absence or presence of VIP. Rat pancreatic tissues were preincubated in KRB buffer solution for 3 min followed by the incubation with or without VIP for the indicated times. Determination of protein output was carried out as described in Materials and Methods. Results represent mean±S.E. of 9 separate experiments. *: $p<0.05$ vs. control (two-tailed Student's t-test)
○—○: Control, ●—●: VIP 2×10^{-7} M/0.5 g wet tissue

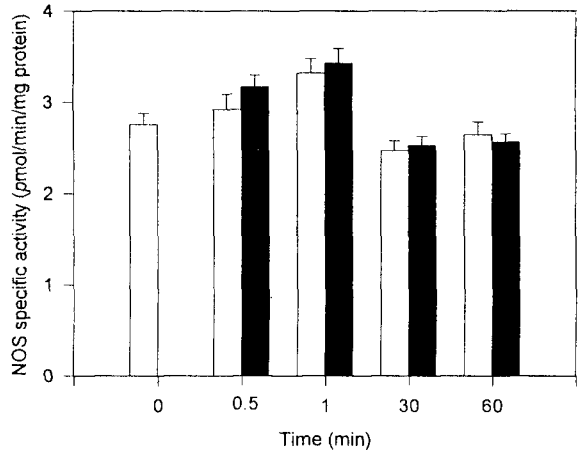

Fig. 3. Effect of time course on the NOS activity in rat pancreatic tissue in the absence or presence of VIP. Rat pancreatic tissues were incubated with or without VIP for the indicated times following the preincubation in KRB buffer solution for 3 min. NOS activity was determined using an assay based on the conversion of ^{3}H-L-arginine to ^{3}H-L-citrulline as described in Materials and Methods. Results represent mean±S.E. of 7-10 separate experiments.
□: Control, ■: VIP (2×10^{-7} M/0.5 g wet tissue)

cubation time point (Fig. 2). These results are consistent with the increase in pancreatic enzyme secretion by VIP-treatment (Meyer-Alber *et al.*, 1994).

In our laboratory, it was revealed that NO is not only involved in the enzyme secretion of rat pancreatic tissues in response to CCK-PZ, but mediates the response to CCK-PZ through cGMP (Seo *et al.*, 1995). On the other hand, VIP has been shown to potentiate the effect of secretagogues that increase intracellular calcium, such as acetylcholine and CCK-PZ (Meyer-Alber *et al.*, 1994). To examine whether NO synthesis step is the point where these distinct pathways funnel into the enzyme secretion, NOS activity was measured at the indicated time. In VIP-treated rat pancreatic tissues, VIP caused the small, but not significant increase in NOS activity at all incubation time points (Fig. 3). Therefore, it could be expected that the interaction point might be the cGMP synthesis step, downstream of NOS synthesis. However, the cGMP level was also not affected by VIP treatment for various times (Fig. 4).

In order to further confirm the previous results, we examined whether the addition of an inhibitor of NOS, L-NAME changes the VIP-induced pancreatic

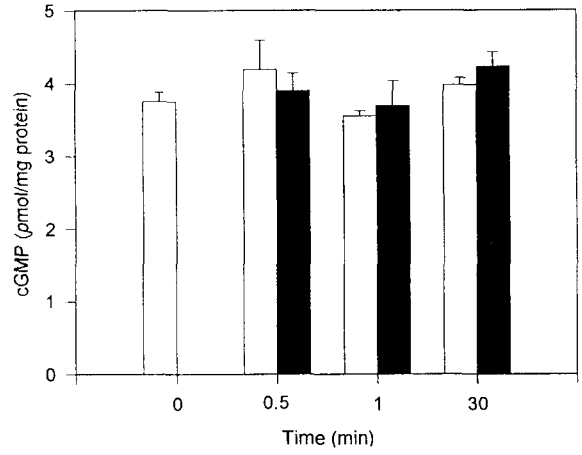

Fig. 4. Effect of time course on the cGMP level in rat pancreatic tissue in the absence or presence of VIP. Rat pancreatic tissues were preincubated in KRB buffer solution for 3 min, and then incubated with or without VIP for indicated times. cGMP level was determined using immunoassay kit as described in Materials and Methods. Results represent mean±S.E. of 6 separate experiments.
□ : Control, ■ : VIP: (2×10^{-7} M/0.5 g wet tissue)

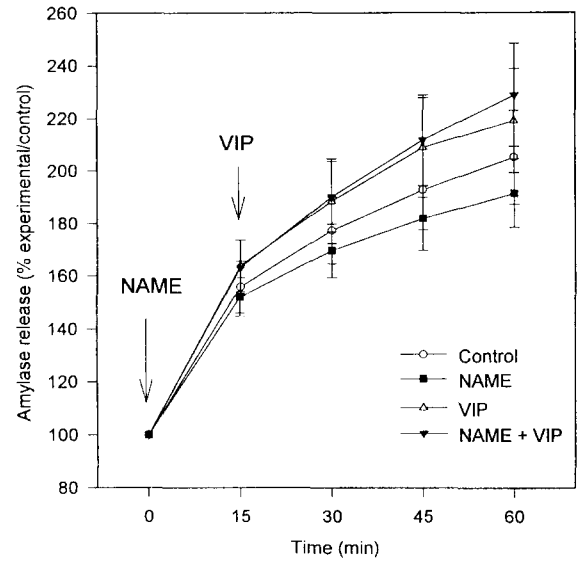

Fig. 5. Inhibitory effect of L-NAME on the amylase release into the incubation medium of rat pancreatic tissue in the absence or presence of VIP. Rat pancreatic tissues were preincubated in KRB buffer solution for 3 min, and then incubated with or without L-NAME (1 mM/0.5 g wet tissue) for 15 min and, subsequently, VIP (2.0×10^{-7} unit/0.5 g wet tissue) was added. Results represent mean±S.E. of 6 separate experiments.
NAME: 1 mM, VIP: 2×10^{-7} M/0.5 g wet tissue

enzyme secretion. Incubation of rat pancreatic tissues with an arginine derivative inhibitor of NOS, L-NAME (1 mM/0.5 g wet tissue), had no effect on the VIP-stimulated amylase release (Fig. 5). On the other hand, VIP-treated groups (VIP, L-NAME+VIP group) have a tendency to increase amylase release compared with the group not treated with VIP (control, L-NAME group).

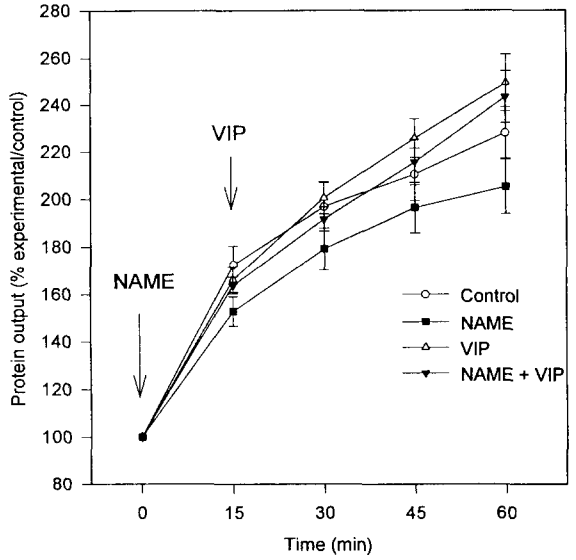

Fig. 6. Inhibitory effect of L-NAME on the protein output into the incubation medium of rat pancreatic tissue in the absence or presence of VIP. Rat pancreatic tissues were preincubated in KRB buffer solution for 3 min, and then incubated with or without L-NAME (1 mM/0.5 g wet tissue) for 15 min and, subsequently, VIP (2.0×10^{-7} unit/0.5 g wet tissue) was added. Results represent mean±S.E. of 6 separate experiments.
NAME: 1 mM, VIP: 2×10^{-7} M/0.5 g wet tissue

Similarly, the VIP-stimulated protein output was not affected by pretreatment of rat pancreatic tissues with L-NAME (Fig. 6).

DISCUSSION

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, CCK-PZ, and bombesin cause changes in intracellular Ca^{2+} and digestive enzyme secretion. In pancreatic acinar cells as well as in variety of other tissues, 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. The mechanism and process by which depleted Ca^{2+} stores activate influx is mediated by cGMP (Pandol and Schoeffield-Payne, 1990).

cGMP rise is one of the early events in neurotransmitter or hormone-induced cascade of reactions in pancreatic acinar cells. Increased cGMP has been shown to be associated with stimulation of pan-

creatic enzyme secretion for over 15 years, but its direct role as a mediator in this process has remained controversial. The majority of NO effects under physiological conditions appears to be mediated primarily by the activation of the intracelluar NO receptor guanylyl cyclase (Garbers, 1992) concomitant with a cGMP increase. A line of evidences suggests that NO is involved in regulation of cGMP and Ca^{2+} transport in pancreatic acinar cells (Gukovskaya and Pandol, 1994). Recently, we have revealed that NO produced mediates CCK-PZ-induced enzyme secretion through cGMP formation in rat pancreatic tissues. CCK-PZ caused significant increase in conversion of arginine to citrulline, amount of nitrite/nitrate, amylase release, and cGMP level (Seo *et al.*, 1995). CCK-PZ-stimulated amylase release and the transformation of arginine to citrulline were antagonized by the inhibitor of NOS, L-NAME. The data on the time course of CCK-PZ-induced citrulline formation and the rise of cGMP indicate that NOS and guanylyl cyclase are activated within the first seconds of stimulation. Therefore, activation of NOS 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. In contrast to CCK-PZ, VIP stimulates pancreatic secretion of HCO_3^- into the duodenum (Holst *et al.*, 1979), which is mediated through cAMP. Recent evidence suggests that VIP also induces pancreatic enzyme secretion as CCK-PZ, but its mechanism remains unknown(Meyer-Alber *et al.*, 1994). Also secretin and VIP have been shown to potentiate the effect of secretagogues that increase intracellular calcium, such as acetylcholine and CCK-PZ. Thus, although these two groups of hormones act by distinct pathways, they interact at an unknown step, resulting in potentiation of their individual effects.

In the present study to elucidate the signaling pathway of VIP-induced enzyme secretion, we observed that VIP treatment of rat pancreatic tissue increased amylase release as well as protein output in a dose- and time-dependent manner. However, NOS activity and cGMP synthesis were not affected by VIP treatment as monitored by the NOS activity assay and determining cGMP level, which is further confirmed by NOS-inhibitor study. The results obtained in this work provide evidence that increase in pancreatic enzyme secretion by treatment with VIP has no relationship with NOS activity and cGMP level. The mechanism, by which VIP triggers the pancreatic enzyme secretion could be explained by two possibilities. One possible mechanism might be that the signal emanating from receptor by VIP bypasses the NO/cGMP step, thereby activates the downstream of the NO/cGMP step, resulting in the same effect on the enzyme secretion as CCK-PZ. Another possible mechanism might be that signal transduction of VIP is independent from the signaling pathway triggered by CCK-PZ.

Further studies should clarify the more precise role and mechanism of NO in the overall scheme of secretory regulation in the pancreatic tissues.

REFERENCES CITED

Bennett, M. A., Watt, P. A. C. and Thurston, H., Endothelium-dependent modulation of resistance vessel contraction : studies with N^G-nitro-L arginine methyl ester and N^G-nitro-L-arginine. *Br. J. Pharmacol.*, 107, 616-621 (1992).

Bernfeld, P., Amylases, α and β. *Method in Enzymol.*, 1, 149-158 (1955).

Bradford, M. E., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. *Anal. Biochem.*, 42, 248-254 (1976).

Bredt, D. S. and Snyder, S. H., Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc. Natl. Acad. Sci. USA*, 87, 682-685 (1990).

Christophe. J. P., Frandsen, E. K., Conlon, T. P., Krishna, G. and Gardner, J. D., Action of cholecystokinin, cholinergic agents and A-23187 on accumulation of guanosine 3',5'-monophosphate in dispersed guinea pig pancreatic acinar cells. *J. Biol. Chem.*, 251, 4640 (1976).

Garbers, D. L., Guanylyl cyclase receptors and their endocrine, paracrine, and autocrine ligands. *Cell*, 74, 1-4 (1992).

Gukovskaya, A. and Pandol, S. J., Nitric oxide production regulates cGMP formation and calcium influx in pancreatic acinar cells. *Am. J. Physiol. Gastrointest. Liver Physiol.*, 266, G350-G356 (1994).

Haymovis. A. and Scheele, G. A., Cellular cyclic nucleotides and enzyme secretion in the pancreatic acinar cells. *Proc. Natl. Acad. Sci. U.S.A.*, 73, 156 (1976).

Holst, J. J., Fahrenkrug, J., Knuhtsen, S., Jensen, S. L., Poulsen, S. S. and Nielsen, O. V., Vasoactive intestinal polypeptide (VIP) in the pig pancreas: role of VIPergic nerves in control of fluid and bicarbonate secretion. *Regulatory Peptide*, 8, 245, 245-259 (1984).

Holst, J. J., Schaffalitzky De Muckadell, O. B. and Fahrenkrug, J., Nervous control of pancreatic exocrine secretion in pigs. *Acta physio. scand.*, 105, 33-51 (1979).

Iwatsuki, K., Iijima, F., Yamagishi, F. and Chiba, S., Effects of nitroprusside on pancreatic secretion and cyclic nucleotide concentration in the dog pancreas. *European J. of pharmacol.*, 123, 307-309 (1986).

Kiechle, F. L. and Malinski, T., Nitric Oxide Biochemistry, Pathophysiology and detection. *Am. J. Clin. Pathol.*, 100, 567-575 (1993).

Kruk, Z. L. and Pycock, C. J., *Neurotransmitters and drugs*. Chapman & Hall, 1991.

Meyer-Alber, A., Fetz, I., Waschulewski, I. H., Hö cker, M., Foölsch, U. R. and Schmidt, W. E., Calyculin A, okadaic acid and W-7 interfere with a distal step in pancreatic acinar signal transduction *Biochem. Biophys. Res. Commun.*, 201, 1470-1476 (1994).

Nathan, C. and Xie, Q.-w., Nitric oxide synthases : roles, tolls, and controls. *Cell*, 78, 915-918 (1994).

Pandol, S. J. and Schoeffield-Payne, M. S., Cyclic GMP mediates the agonist-stimulated increase in plasma membrane calcium entry in the pancreatic acinar cells. *J. Biol. Chem.*, 265, 12846-12853 (1990).

Schmidt, H. H. H. W. and Walter, U., NO at work. *cell*, 78, 919-925 (1994).

Seo, D. W., Nam, S. W., Nam, T. K., Lee, Y. J., Ko, Y. K. and Lee, H. W., Effect of cholecystokinin-pancreozymin on the nitric oxide synthase activity and cyclic GMP level in rat pancreatic tissue. *Archives of Pharmacal Research,* 18, 434-439 (1995).

Solomon, T. E., Control of exocrine pancreatic secretion, In *Physiology of the Gastrointestinal tract.* 2, 1173-1208 (1987).

Schulz, I. and Stolz, H. H., The exocrine pancreas: the role of secretagogues, cyclic nucleotides and calcium in enzyme secretion. *Ann. Rev. Physiol.*, 239, 127-156 (1980).