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C-type natriuretic peptide enhances amylase release through NPR-C receptors in the exocrine pancreas

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Sabbatini ME, Rodríguez M, di Carlo MB, Davio CA, Vatta MS, Bianciotti LG. C-type natriuretic peptide enhances amylase release through NPR-C receptors in the exocrine pancreas. *Am J Physiol Gastrointest Liver Physiol* 293: G987–G994, 2007. First published August 16, 2007; doi:10.1152/ajpgi.00268.2007.—Several studies show that C-type natriuretic peptide (CNP) has a modulatory role in the digestive system. CNP administration reduces both jejunal fluid and bile secretion in the rat. In the present study we evaluated the effect of CNP on amylase release in isolated pancreatic acini as well as the receptors and intracellular pathways involved. Results showed that all natriuretic peptide receptors were expressed not only in the whole pancreas but also in isolated pancreatic acini. CNP stimulated amylase secretion with a concentration-dependent biphasic response; maximum release was observed at 1 pM CNP, whereas higher concentrations gradually attenuated it. The response was mimicked by a selective natriuretic peptide receptor (NPR-C) agonist and inhibited by pertussis toxin, strongly supporting NPR-C receptor activation. CNP-evoked amylase release was abolished by U-73122 (PLC inhibitor) and 2-aminoethoxydiphenyl borate (2-APB) [an inositol 1,4,5-triphosphate (IP_3) receptor antagonist], partially inhibited by GF-109203X (PKC inhibitor), and unaltered by ryanodine or protein kinase A (PKA) and protein kinase G (PKG) inhibitors. Phosphoinositide hydrolysis was enhanced by CNP at all concentrations and abolished by U-73122. At 1 and 10 pM, CNP did not affect cAMP or guanosine 3′,5′-cyclic monophosphate (cGMP) levels, but at higher concentrations it increased cGMP and diminished cAMP content. Present findings show that CNP stimulated amylase release through the activation of NPR-C receptors coupled to the PLC pathway and downstream effectors involved in exocytosis. The attenuation of amylase release was likely related to cAMP reduction. The augmentation in cGMP supports activation of NPR-A/NPR-B receptors probably involved in calcium influx. Present findings give evidence that CNP is a potential direct regulator of pancreatic function.

amylase secretion; natriuretic peptide receptors; phospholipase C; protein kinase C

C-TYPE NATRIURETIC PEPTIDE (CNP), a 22-amino acid peptide, is a member of the natriuretic peptide family also including atrial natriuretic peptide (ANF) and B-type natriuretic peptide (BNP). CNP is regarded as an endothelium-derived vasodilator and plays an important role in the control of both vascular tone and vascular remodeling process (4). CNP has considerable sequence homology with ANF and BNP, including a 17-membered disulfide ring structure, essential for the biological activity, but lacks the carboxyl-terminal extension beyond the 17-amino acid ring, which results in weak diuretic and natriuretic properties compared with the other family members (28, 32). Both ANF and BNP are predominantly synthesized and released by cardiac myocytes where they are stored in granules and released by mechanical (atrial stretch) or neuroendocrine stimuli (endothelin-1 or α-adrenergic stimulation) (8). CNP was first reported in the porcine brain (31), but later studies showed that it is synthesized by endothelial cells, as well as by noncardiac tissues (15).

Molecular cloning techniques revealed the existence of three natriuretic peptide receptors (NPR-A, NPR-B, and NPR-C) that are widely distributed and display distinct affinities for the members of the natriuretic peptide family (1). CNP preferentially binds to NPR-B and NPR-C (32). The NPR-A and NPR-B receptors, which mediate most of the renal and cardiovascular effects of the natriuretic peptides, are membrane guanylyl cyclases with a tyrosine kinase-like domain (34). The NPR-C receptor displays similar affinity for all the natriuretic peptides but exhibits distinct structural properties. It was originally considered a clearance receptor, but later studies revealed that it is a G protein-coupled receptor (GPCR) (2). Its intracellular domain is devoid of guanylyl cyclase and kinase activities, and it is coupled to adenylyl cyclase (AC) inhibition through an inhibitory guanine nucleotide regulatory protein (G_i) and/or to phospholipase C (PLC) activation (2, 17). This receptor subtype coupled to G_{i-1} and G_{i-2} is the predominant natriuretic peptide receptor in visceral and vascular smooth muscle cells (17). The mRNAs of natriuretic peptide receptors have been shown in diverse tissues as the heart, brain, kidney, stomach, and salivary glands among others (2, 12, 15, 17, 28, 34). Although previous binding as well as functional studies support the expression of the three natriuretic peptide receptors in the pancreas (10, 24, 27), their mRNAs have not been reported in this gland so far.

CNP plays a relevant role in blood pressure regulation, but it also participates in the modulation of digestive function (4, 35). We have previously reported that CNP is involved in the central as well as peripheral regulation of bile secretion in the rat (25, 26). The intravenous administration of CNP reduces bile secretion by decreasing bile acid output through the activation of NPR-C receptors (26). When the peptide is applied to the brain, it also reduces bile flow without the participation of the autonomic nervous system, supporting the role of peptidergic pathways (25). CNP not only reduces bile secretion, but it also diminishes intestinal secretion (16). However, when CNP is centrally applied, it stimulates pancreatic

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fluid and protein output through vagal pathways by the activation of central NPR-A and/or NPR-B receptors (23).

In the present study we sought to establish the effect of CNP on amylase release in isolated pancreatic acini and the receptors as well as the intracellular signaling mechanisms involved. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis showed the expression of the three natriuretic peptide receptors not only in the whole pancreas but also in isolated pancreatic acini. Furthermore, CNP stimulated amylase secretion through NPR-C receptors coupled to the PLC pathway, showing a concentration-dependent biphasic response similar to that elicited by known pancreatic secretagogues. The maximum amylase release was achieved at 1 pM CNP; then higher concentrations attenuated the response in parallel with a decrease in cAMP content and an increase in guanosine 3′,5′-cyclic monophosphate (cGMP) levels.

MATERIALS AND METHODS

Sprague-Dawley strain rats weighing between 250 and 300 g bred in Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, were used in the experiments. They were housed in steel cages and maintained at 22–24°C in a controlled room with 12:12-h light-dark cycle (light from 0700 to 1900). All experimental protocols were approved by the Animal Care Committee of the Facultad de Farmacia y Bioquimica, Universidad de Buenos Aires. All experiments were conducted following the recommendations of the National Institutes of Health guidelines for the care and use of laboratory animals (NIH Publication N85-23, 1985, revised 1996).

The following compounds were used: CNP, CCK, [D-p-Cl-Phe^6-Leu^{17}]VIP, and Des(Gln^{18}, Ser^{19}, Gln^{20}, Leu^{21}, Gly^{22}) ANP-(4-23)-NH_2 [cANP (4-23 amide)] (American Peptides, Sunnyvale, CA); H-89, KT-5823, U-73122, 2-aminoethoxydiphenyl borate (2-APB), ryanodine, and GF-109203X (Calbiochem, San Diego, CA); N^{ω}-nitro-L-arginine methyl ester (L-NAME), 3-isobutyl-1-methylxanthine (IBMX), atropine sulphate, hyaluronidase, α-chymotrypsin, type V collagenase, cGMP antiserum, bovine serum albumin (BSA), pertussis toxin, proglumide, and carbamylcholine chloride (carbachol) (Sigma, St. Louis, MO); [^{3}H]cAMP and Myo [^{3}H]inositol (Amersham Biosciences, Buckinghamshire, UK). Other reagents were of analytical or molecular biology quality and obtained from standard sources.

Expression of Natriuretic Peptide Receptors

The expression of natriuretic peptide receptors (NPR-A, NPR-B, and NPR-C) in rat brain, heart, pancreas, and pancreatic acini was assessed by RT-PCR. As the gene expression of natriuretic peptide receptors was reported in brain and heart, these tissues were used as positive controls (2, 28). The following primers were used: rat NPR-A: 5′-GCGTGGTAGATGGACGTTTT-3′ (sense), 5′-GGCAATTTCCTGAAGGATGA-3′ (antisense); rat NPR-B: 5′-TGTACCCTGCTGCTTCTGTG-3′ (sense), 5′-TCTGTGCGAGCATCCAGATA-3′ (antisense); rat NPR-C: 5′-CTGGTCTACAGCGACGACAA-3′ (sense), 5′-TGATTCTCCGAATGGTGTCA-3′ (antisense) (Invitrogen, Carlsbad, CA). The primers were designed with Invitrogen Oligoperfect Designer based on gene sequences obtained from the GenBank NCBI Sequence Viewer (http://www.ncbi.nlm.nih.gov). Total RNA was isolated from heart, brain, whole pancreas, and isolated pancreatic acini by using TRIzol and RNeasy spin columns. RNA quality and quantity was assessed by 1% agarose gel electrophoresis and UV spectrometry (OD260/OD280 $>$ 1.7). One microgram of total RNA was reverse transcribed by using *Taq* Man reverse transcription reagents with random hexamers as primers (Roche Diagnostics, Branchburg, NJ). For NPR-A, NPR-B, and NPR-C amplification, total RNA template was denatured by heating at 90°C for 5 min and annealed by slow cooling to 25°C. Reverse transcription was carried out by the addition of 200 U reverse transcriptase (Roche Diagnostics) followed by incubation for 30 min at 48°C in PCR buffer (Roche Diagnostics) in a total volume of 50 μl. Reverse transcription was terminated by heat-inactivating the reverse transcriptase for 10 min at 95°C. The PCR was carried out in the same reaction mixture containing forward and reverse primers (125 ng) and 3.5 U Taq polymerase (Roche Diagnostics). PCR consisted of denaturation at 95°C for 10 s, annealing at 68°C for 30 s, and extension at 72°C for 30 s, for 35 cycles, in an automated thermocycler (Eppendorf Mastercycler). A negative control was run in the absence of reverse transcriptase to check the specificity of the amplification. The PCR products were then submitted to electrophoresis on 1.5% ethidium bromide-stained agarose gel, and blots were developed by Gel Doc XR System.

Studies In Isolated Pancreatic Acini

Preparation of isolated pancreatic acini. Isolated pancreatic acini were obtained as previously described (24, 27, 36). The basic medium used for the isolation and incubation of pancreatic acini was a modified Krebs-Henseleit bicarbonate buffer (KHB) of the following composition: 118 mM NaCl, 4.7 mM KCl, 1 mM $NaHPO_4$, 1.1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 2.5 mg/ml D-glucose, minimal Eagle's medium amino acid supplement, and 0.1 mg/ml soybean trypsin inhibitor. The medium was equilibrated with 95% O_2-5% CO_2 and adjusted to pH 7.35. Briefly, pancreatic tissue was obtained from overnight fasted rats. The dissociation medium (KHB containing 60–70 U/ml purified collagenase, 25 μg/ml chymotrypsin, and 1.8 mg/ml hyaluronidase) was injected into the pancreatic parenchyma. Tissue was then incubated for 10 min and for 45 min in fresh KHB with shaking at 120 cycles/min followed by sucking up and down with polypropylene pipettes to dissociate the acini. After filtration through a 150 μm nylon mesh, acini were purified by centrifugation at 50 *g* for 4 min in a KHB without enzymes and containing 4% BSA. Pancreatic acini were finally suspended in a KHB medium containing 1% BSA.

Amylase secretion in isolated pancreatic acini. Acini were preincubated in the suspension medium at 37°C and gassed with 95% O_2-5% CO_2 for 30 min and then incubated in fresh medium for 30 min in the presence of CNP, cANP (4-23 amide), CCK, and carbachol. Acini were also pretreated with 1 μM atropine (muscarinic antagonist), 10 μM [D-p-Cl-Phe^6-Leu^{17}]VIP (VPAC antagonist), 100 μM proglumide (CCK antagonist), 10 μM L-NAME (nitric oxide synthase inhibitor), 100 μM 2-APB [an inositol 1,4,5-triphosphate (IP_3) receptor antagonist], 50 μM ryanodine (ryanodine receptor inhibitor), 10 μM U-73122 (PLC inhibitor), 100 nM GF-109203X (PKC inhibitor), 10 μM H-89 (PKA inhibitor), and 2 μM KT-5823 (PKG inhibitor) before the addition of 1 pM CNP. Acini were then centrifuged for 2 min, and amylase activity was determined by using 1,4-α-nitrophenylmaltoheptaoside as substrate in supernatants and pellets previously lysed with 0.1% Triton X-100 (22). Results were expressed as percentage of total amylase. Basal values corresponded to nonstimulated amylase release. In other experiments, freshly isolated pancreatic acini were incubated in the presence or in the absence of pertussis toxin, G_i protein inhibitor, for 2 h at 37°C and gassed with 95% O_2-5% CO_2 (23). Tissues were then washed twice in the suspension medium and incubated with 1 pM CNP. Amylase was determined as detailed above. Results were expressed as percentage of basal amylase release, taking basal value as 100%.

cAMP and cGMP determination. The accumulation of cAMP was assessed as previously described (7, 24). Briefly, acini were preincubated for 30 min at 37°C and then incubated for 3 min in fresh Krebs solution containing 1 mM IBMX and then for 12 min with CNP. Experiments were also carried out in the presence of 10 μM U-73122, 100 nM GF-109203X, 2 μM KT-5823, 10 μM L-NAME to inhibit PLC, PKC, PKG, and nitric oxide synthase activities, respectively. Inhibitors were added 5–10 min before the addition of the peptide. Reaction was stopped by homogenization in ice-cold ethanol and centrifuged for 15 min at 1,200 *g*. The supernatant was dried and the

residue suspended for cAMP determination that was assessed by competition of [^{3}H]cAMP for PKA as previously detailed (7). The content of cGMP was determined by radioimmunoassay as previously detailed (29). All cyclic nucleotides were expressed as picomoles per milligram protein.

Phosphoinositide Hydrolysis Measurement

Phosphoinositide turnover was determined as previously detailed (5, 27). Briefly, isolated pancreatic acini were incubated in KHB containing 10 mM LiCl and 4 μCi/ml myo-[^{3}H]inositol for 120 min and gassed with 95% O_2-5% CO_2. Thirty minutes before the end of the incubation period 1 pM and 10 and 100 nM CNP or cANP (4-23 amide) were added. Acini were then washed with nonradioactive medium and homogenized in chloroform-methanol (1:2 vol/vol). Phases were separated by the addition of chloroform and water, and the upper phase was applied to an anion exchange column (Bio-Rad X8 resin, 100–200 mesh, formate form) followed by the addition of unlabeled myoinositol. Columns were washed and then eluted with 1 M ammonium formate and 0.1 M formic acid. Total inositol phosphates were expressed as percentage of control.

Statistical Analysis

Results are expressed as the means ± SE. The statistical analysis was performed by ANOVA followed by the Student-Newman-Keuls test. A P value of 0.05 or less was considered statistically significant.

RESULTS

Binding, pharmacological, and functional studies support the idea that natriuretic peptide receptors are expressed in the pancreas (10, 24, 27). In the present work we demonstrated by RT-PCR that NPR-A, NPR-B, and NPR-C were all expressed, not only in the whole pancreas, but also in isolated pancreatic acini (Fig. 1). NPR-A, NPR-B, and NPR-C primers yielded products of the expected size (203, 223, and 208 bp, respectively). In addition, a semiquantitative approach was also performed by analyzing the PCR products at 28 and 32 cycles to compare the relative abundance of these transcripts between the whole pancreas and pancreatic acini. As no differences were found, it strengthens the identification of natriuretic peptide receptors on acinar cells (data not shown).

CNP concentration dependently stimulated amylase release from isolated pancreatic acini showing a biphasic response similar to that of the pancreatic secretagogue CCK (Fig. 2). CNP at 0.1 pM enhanced amylase release, reaching a maximum (230%) at 1 pM CNP; at higher concentrations, the stimulatory response was gradually decreased, although it remained elevated compared with non-stimulated or basal release (3.5 ± 0.3%) (Fig. 2).

The selective agonist of NPR-C receptors, cANP (4-23 amide), mimicked CNP effect showing a similar biphasic response, although the peak was achieved at a higher concentration than that of CNP (Fig. 3). However, the maximum amylase release was similar for both CNP and the selective agonist (Fig. 3, inset). The incubation of acini with CNP and cANP (4-23 amide) combined did not further increase the enzyme output (data not shown). Furthermore, pretreatment of the acini with pertussis toxin abolished CNP-evoked amylase release (Fig. 4). These findings strongly support the idea that the increase in amylase output induced by CNP was mediated by NPR-C receptors.

We next conducted experiments to investigate whether muscarinic, CCK, and VPAC receptors or nitric oxide were involved in CNP response. Blockade of muscarinic, CCK, and VPAC receptors or the inhibition of nitric oxide synthase failed to affect basal or CNP-evoked pancreatic amylase release, supporting the idea that neither these receptors nor nitric oxide were involved in CNP stimulatory effect on amylase output (Fig. 5).

The major pancreatic secretagogues that evoke amylase release signal through the activation of PLC and downstream effectors that are intimately related to the exocytotic process (37, 38). As CNP-evoked amylase release was mediated by NPR-C receptors, experiments were performed to determine the intracellular signaling activated by CNP. Acini were pretreated with different inhibitors before the addition of the peptide. Results showed that CNP-induced amylase release was abolished in the presence of U-73122 (PLC inhibitor), and it was

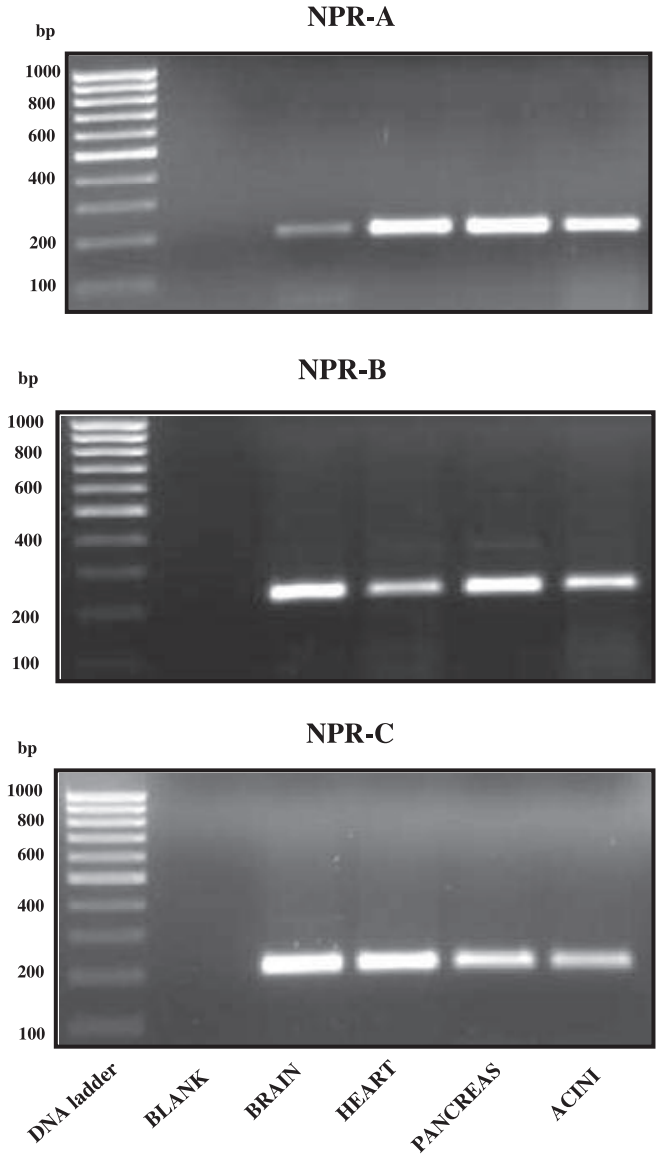

Fig. 1. Expression of natriuretic peptide receptors. The expression of NPR-A, NPR-B, and NPR-C was assessed in brain, heart, and whole pancreas as well as in isolated pancreatic acini by RT-PCR as detailed in MATERIALS AND METHODS. NPR-A, NPR-B, and NPR-C primers yielded products of the expected size (203, 223, and 208 bp, respectively).

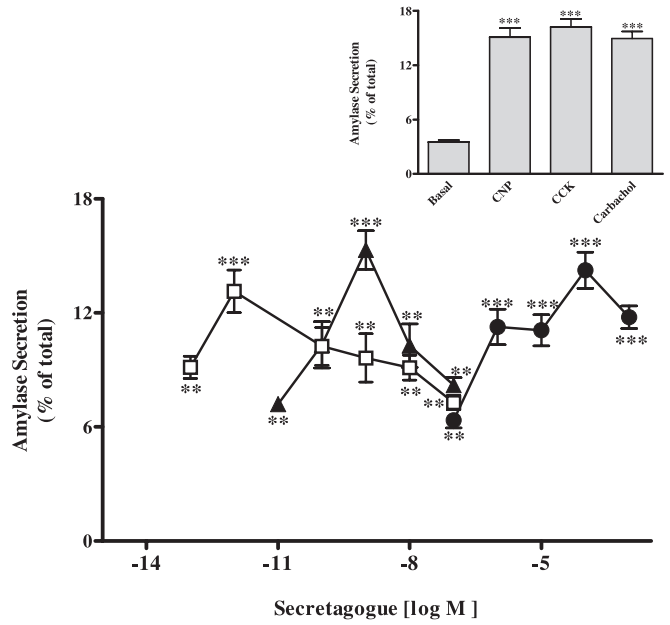

Fig. 2. Amylase release induced by C-type natriuretic peptide (CNP) (□), CCK (▲) and carbachol (●). Isolated pancreatic acini were incubated for 30 min in the presence of CNP (0.1 pM–100 nM), CCK (10 pM–100 nM), and carbachol (100 nM–1 mM). Amylase was measured and expressed as percentage of total amylase. Number of experiments: 10–12. $^{**}P < 0.01$; $^{***}P < 0.001$ vs. basal amylase release. *Inset:* maximal amylase secretion in isolated acini induced by CNP, CCK, and carbachol. $^{***}P < 0.001$ vs. basal amylase release.

partially inhibited by GF-109203X (PKC inhibitor) (Fig. 6*A*) and 2-APB (IP_3 receptor inhibitor). However, CNP-evoked amylase secretion resulted unaltered by pretreatment with H-89 (PKA inhibitor) or KT-5823 (PKG inhibitor) (data not shown), supporting the idea that CNP response was mediated by the activation of the PLC pathway and downstream effectors as observed for other secretagogues like CCK and carbachol. Ryanodine pretreatment did not affect basal or CNP-evoked amylase release, showing that CNP did not stimulate ryanodine

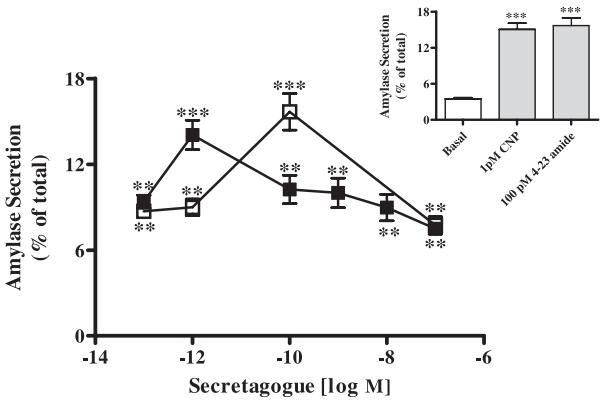

Fig. 3. Effect of CNP (■) and cANP (4-23 amide) (NPR-C receptor agonist) (□) on amylase secretion. Isolated pancreatic acini were incubated for 30 min in the presence of CNP or cANP (4-23 amide). Amylase was measured and expressed as percentage of total amylase. Number of experiments: 10–12. $^{**}P < 0.01$; $^{***}P < 0.001$ vs. basal amylase release. *Inset*: maximal amylase secretion in isolated acini induced by CNP and cANP (4-23 amide). $^{***}P < 0.001$ vs. basal amylase release.

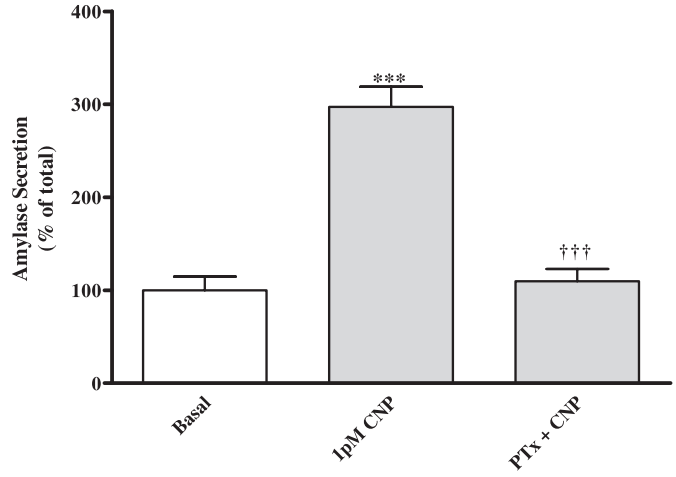

Fig. 4. Effect of pertussis toxin (PTx) pretreatment on CNP-evoked amylase release. Isolated pancreatic acini were pretreated with pertussis toxin as described in MATERIALS AND METHODS followed by incubation with 1 pM CNP. Amylase was determined and expressed as percentage of basal amylase release (100%). Number of experiments: 8–10. $^{***}P < 0.001$ vs. basal amylase release; $^{\dagger\dagger\dagger}P < 0.001$ vs. CNP.

receptors, which are involved in the calcium signal propagation across the acinar cell (30) (Fig. 6*B*).

To support the activation of PLC by CNP, phosphoinositide turnover was assessed. CNP concentration dependently enhanced phosphoinositide hydrolysis in isolated pancreatic acini, but the increase was abolished in the presence of U-73122 indicating PLC activation (Fig. 7). The response was mimicked by the selective agonist cANP (4-23 amide) (data not shown), further supporting NPR-C activation.

As the NPR-C receptor is also coupled to AC inhibition (2), cAMP content was assessed. CNP at 1 and 10 pM did not

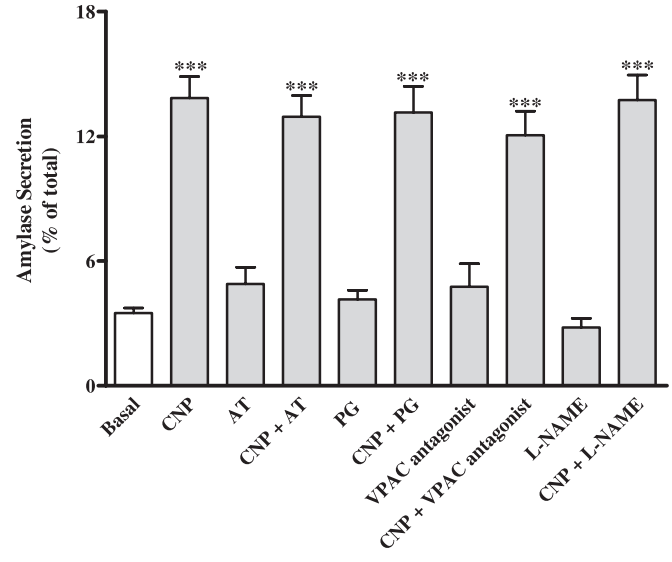

Fig. 5. CNP-evoked amylase release in the presence of muscarinic, CCK, and VIP antagonists and nitric oxide synthase inhibition. Isolated pancreatic acini were pretreated with 1 μM atropine (AT) (muscarinic antagonist), 100 μM proglumide (PG) (CCK antagonist), 10 μM [D-p-Cl-Phe^6-Leu^{17}]VIP (VPAC antagonist), or 10 μM N^{ω}-nitro-L-arginine methyl ester (L-NAME) (NO synthase inhibitor) and then exposed to 1 pM CNP. Amylase was assessed and expressed as percentage of total amylase. Number of experiments: 8–10. $^{***}P < 0.001$ vs. basal amylase release.

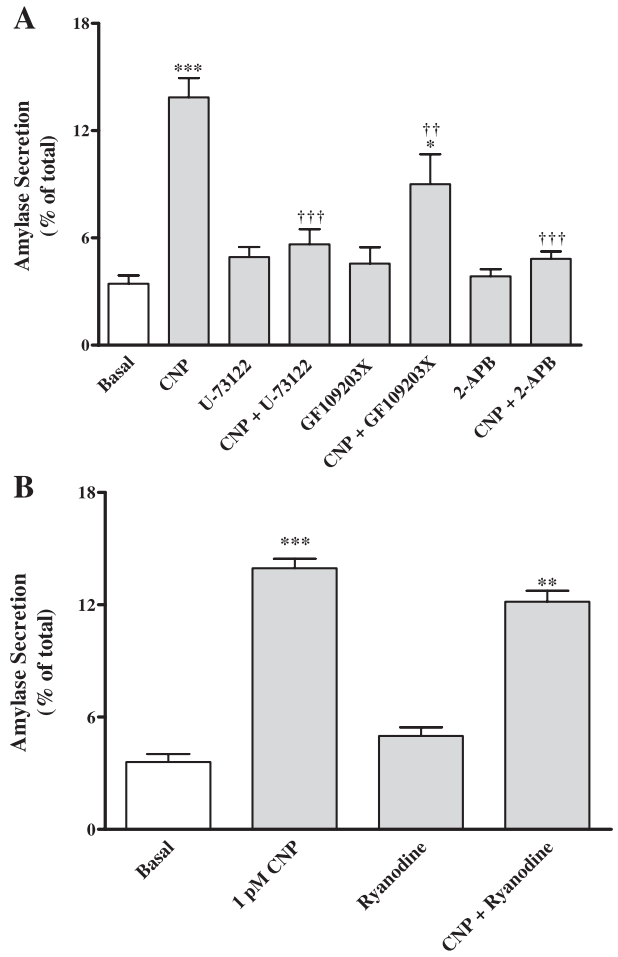

Fig. 6. *A*: effect of PLC, PKC, and IP_3 inhibitors on CNP-evoked amylase release. Isolated pancreatic acini were pretreated with 10 μM U-73122 (PLC inhibitor), 100 nM GF-109203X (PKC inhibitor), or 100 μM 2-aminoethoxydiphenyl borate (2-APB) [an inositol 1,4,5-trisphosphate (IP_3) receptor antagonist] and then incubated with 1 pM CNP. Amylase was measured and expressed as percentage of total amylase. Number of experiments: 8–10. $*P < 0.05$; $***P < 0.001$ vs. basal amylase release; $††P < 0.01$; $†††P < 0.001$ vs. CNP. *B*: effect of ryanodine receptor inhibition on CNP-evoked amylase release. Isolated pancreatic acini were preincubated with 50 μM ryanodine and then incubated with 1 pM CNP. Amylase was measured and expressed as percentage of total amylase. Number of experiments: 6–8. $**P < 0.01$; $***P < 0.001$ vs. basal amylase release.

modify basal cAMP intracellular levels but at 1, 10, and 100 nM it concentration dependently reduced them (Fig. 8). The inhibition of cAMP was abolished by U-73122 (PLC inhibitor) and GF-109203X (PKC inhibitor) (Fig. 9, *A* and *B*, respectively). Pretreatment with either KT-5823 or L-NAME failed to affect CNP response (data not shown).

Cyclic GMP levels were also assessed in the presence of CNP because, although the peptide binds to the NPR-C receptor, it is also a ligand for the NPR-B receptor (28), which is expressed in pancreatic acini as shown in the present study. In addition, increases in cGMP content induced by this natriuretic peptide have been reported in various tissues and cell types (28). The intracellular levels of cGMP were not affected by CNP at 1 and 10 pM but were increased by 1, 10, and 100 nM CNP, showing a concentration biphasic effect, reaching the maximum at 10 nM (Fig. 10).

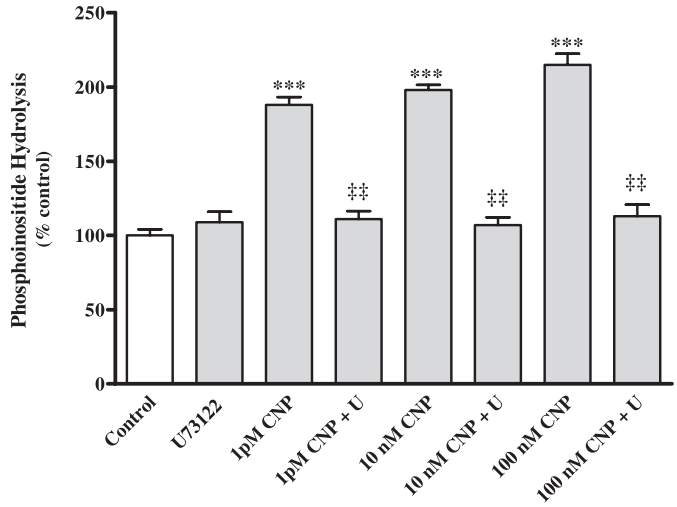

Fig. 7. Effect of CNP on phosphoinositide turnover. Isolated pancreatic acini were incubated with 1 pM, 10 and 100 nM CNP or pretreated with 10 μM U-73122 (U) (PLC inhibitor) before incubation with CNP. Phosphoinositide hydrolysis was determined as detailed in MATERIALS AND METHODS and expressed as percentage of control. Number of experiments: 6–8. $***P < 0.001$ vs. control; $‡‡P < 0.01$ vs. CNP.

DISCUSSION

Pancreatic secretion is hormonally and neurally regulated with a large number of neuropeptides involved in this complex regulatory process (37, 38). Hormones such as CCK, neuropeptides including VIP, and neurotransmitters, especially acetylcholine released upon cholinergic stimulation, induce the fusion of zymogen granules with the apical membrane of acinar cells, resulting in the release by exocytosis of digestive enzymes into the acinar lumen (37, 38).

The major findings of the present study were that all natriuretic peptide receptors were expressed in the exocrine pancreas and that CNP concentration dependently stimulated amylase release through the activation of NPR-C receptors coupled to the PLC pathway and downstream effectors. The maximum amylase release was observed at 1 pM CNP; then higher concentrations of the peptide gradually attenuated the enzyme

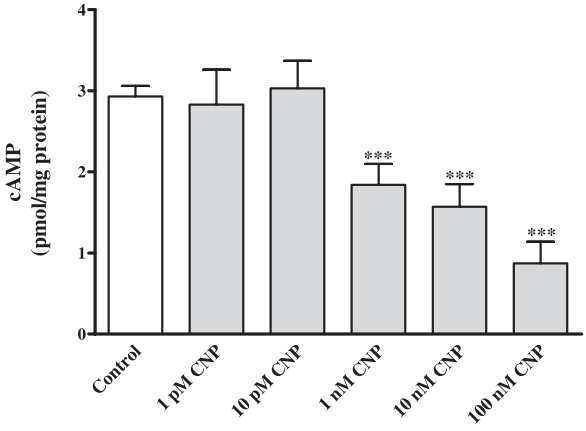

Fig. 8. Cyclic AMP intracellular levels in the presence of CNP. Isolated pancreatic acini were incubated in the presence of CNP (1, 10 pM; 1, 10, and 100 nM). Cyclic AMP was determined as detailed in MATERIALS AND METHODS, and results were expressed as pmol/mg protein. Number of experiments: 8–10. $***P < 0.001$ vs. control.

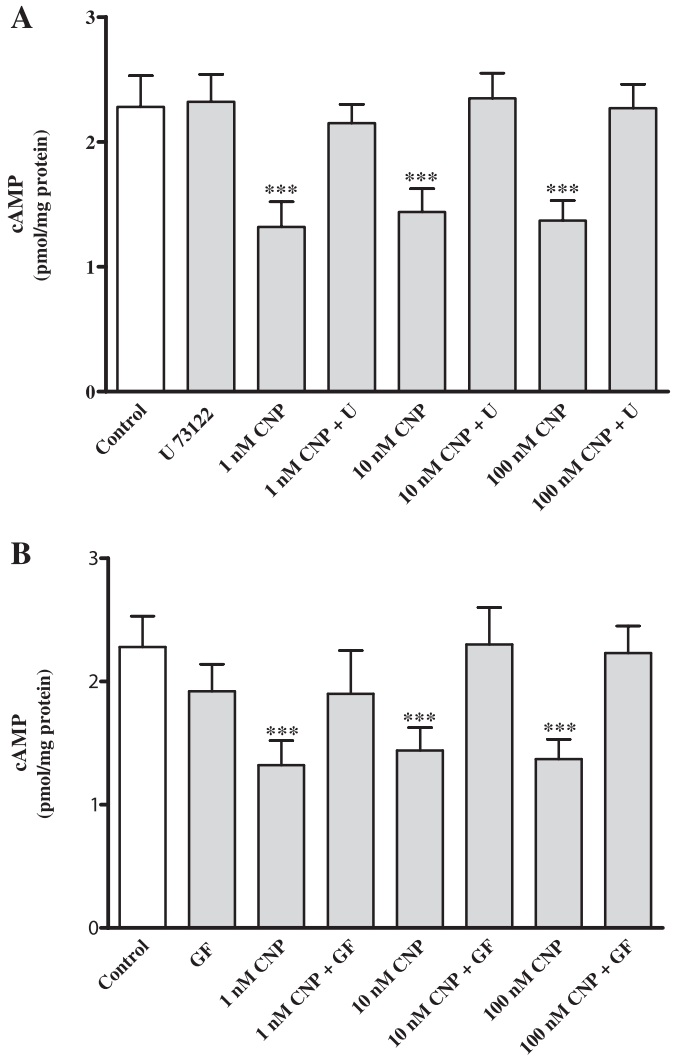

Fig. 9. Effect of PLC (*A*) and PKC (*B*) inhibitors on CNP-evoked reduction of cAMP levels. Isolated pancreatic acini were pretreated with 10 μM U73122 (U) (PLC inhibitor) or 100 nM GF-109203X (GF) (PKC inhibitor) followed by incubation with CNP. Cyclic AMP was determined as detailed in MATERIALS AND METHODS, and results expressed as pmol/mg protein. Number of experiments: 9–10. ***$P < 0.001$ vs. control.

output. CNP showed a biphasic response or restricted stimulation, similar to that evoked by classic secretagogues, where enzyme release is increased, reaching a peak, after which higher secretagogue concentrations gradually decrease the response (3). CNP reached the maximum amylase release at a lower concentration than CCK or carbachol.

CNP enhanced amylase release through NPR-C receptor activation. The selective agonist of NPR-C receptors, cANP (4-23 amide), mimicked CNP stimulatory effect and also exhibited a biphasic response, but the highest output of the enzyme, although of a similar magnitude, was reached at a higher concentration. Since the NPR-C receptor is coupled through G_i proteins to the inhibition of AC and/or the activation of PLC, whereas NPR-A and NPR-B are membrane guanylyl cyclases with a tyrosine kinase-like domain (2, 33), the participation of NPR-C receptors was further supported by pretreatment of the acini with pertussis toxin, which abolished CNP-evoked amylase release. Moreover, combined incubation of pancreatic acini with 1 pM CNP and the selective agonist of NPR-C did not further increase the enzyme output. These findings exclude the participation of NPR-A and NPR-B receptors in CNP-evoked amylase release.

Although CNP-evoked amylase release involved NPR-C receptors, the participation of CCK and muscarinic receptors was investigated. Pretreatment of pancreatic acini with atropine or proglumide failed to affect basal or CNP-evoked amylase output, showing that neither CCK nor muscarinic receptors were involved in CNP response. VIP is a neuropeptide released from the enteric nervous system and intrapancreatic ganglia that, through the activation of VPAC-1 and VPAC-2 receptors, enhances pancreatic fluid secretion (37). Blockade of VPAC receptors with [D-p-Cl-Phe^6-Leu^{17}]VIP modified neither basal nor CNP-stimulated amylase release, supporting the idea that VIP was not involved in CNP effect. Nitric oxide is also produced in the exocrine pancreas, and blockade of nitric oxide synthase has been reported to reduce CCK-, carbachol-, and secretin-stimulated pancreatic secretion, as well as to inhibit enzyme secretion (13). In the present study, blockade of nitric oxide synthase failed to modify amylase release either in basal conditions or when stimulated by CNP, thus excluding the participation of nitric oxide in CNP response.

The major intracellular pathway involved in pancreatic enzyme release is PLC activation, which produces IP_3 and diacylglycerol (DAG) (37, 38). IP_3 induces calcium release from intracellular stores, whereas DAG activates PKC, which in turn phosphorylates diverse proteins involved in the exocytotic process. Intracellular free calcium is the primary signaling molecule in acinar cells, as it is the main driver of digestive enzyme secretion (37, 38). The initial calcium increase produced upon secretagogue stimulation leads to an oscillatory form of calcium increase in the apical pole of acinar cell, consistent with IP_3 receptor localization. This calcium signal is then propagated across the cell by ryanodine receptors (30). As CNP-evoked amylase release was mediated by NPR-C receptors, the signaling pathways coupled to this receptor subtype were studied. The stimulatory effect of CNP on amylase secretion was mediated by the activation of the PLC/IP_3

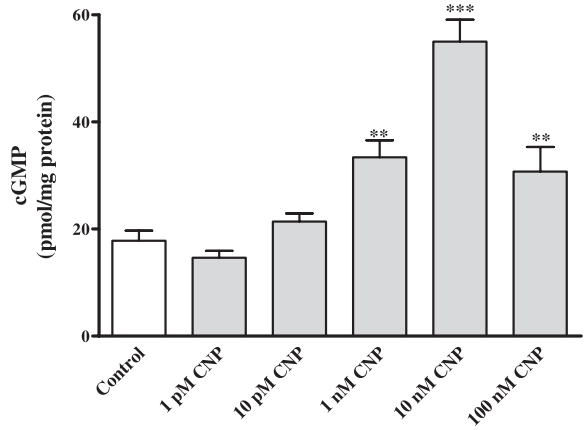

Fig. 10. Cyclic GMP intracellular levels in the presence of CNP. Isolated pancreatic acini were incubated with 1, 10 pM; 1 nM, 10 nM, and 100 nM CNP and guanosine 3′,5′-cyclic monophosphate (cGMP) determined as detailed in MATERIALS AND METHODS. Results were expressed as pmol/mg protein. Number of experiments: 6–8. **$P < 0.01$; ***$P < 0.001$ vs. control.

pathway since the effect was abolished in the presence of a PLC inhibitor and an IP_3 receptor antagonist. The latter finding is in accordance with previous studies showing that pretreatment with 2-APB completely prevented acetylcholine-induced calcium oscillations in pancreatic acinar cells (39). Furthermore, ryanodine did not affect basal or CNP-evoked amylase release. The ryanodine receptor responds to calcium with calcium-induced calcium release and is involved in the propagation of the calcium signal but not in its initiation (30). Previous studies showed that ryanodine modifies calcium intracellular pattern without affecting acetylcholine-stimulated enzyme secretion (18). Present findings show that CNP through NPR-C receptors stimulated amylase output coupled to the activation of the PLC/IP_3 pathway, increasing intracellular calcium levels through IP_3 receptor activation. This was further confirmed by the observation that CNP increased phosphoinositide turn over, the effect being abolished by a PLC inhibitor and mimicked by cANP (4-23 amide), supporting the idea that PLC activation was mediated by NPR-C activation. Nevertheless, the PLC/DAG/PKC pathway was also involved because CNP-evoked amylase secretion was partially inhibited by PKC inhibition. Current literature strongly supports the idea that pancreatic exocytosis can be evoked only by changes in cytosolic calcium and that cAMP, as well as PKC, participate in the recruitment of zymogen granules sensitive to calcium, which are located farther from the apical luminal membrane (14, 37, 38). The partial inhibition of CNP-evoked amylase release by a PKC inhibitor suggests that the peptide also stimulates PKC-induced recruitment of zymogen granules sensitive to calcium.

CNP response was not affected by PKA or PKG inhibitors. However, the second messengers that stimulate these kinases were assessed for various reasons. Both NPR-A and NPR-B are expressed in pancreatic acini as shown in the present study. In addition, CNP is the natural ligand of NPR-B receptors and increases cGMP content in diverse tissues and cell types (28). Furthermore the NPR-C receptor may also be coupled to AC inhibition besides PLC activation (2). CNP at 1 and 10 pM modified neither cAMP nor cGMP content in the acini, supporting the idea that, at these concentrations, the PLC/IP_3 and PLC/DAG/PKC pathways were the only intracellular signaling involved in CNP-evoked amylase release. However, the peptide at 1, 10, and 100 nM reduced cAMP levels and increased cGMP content. The reduction of cAMP at 1, 10, and 100 nM CNP may be likely related to the attenuation of amylase output observed at those concentrations of the peptide. It is known that acute activation of $G_{\alpha i/0}$ coupled receptors inhibits cAMP accumulation (6, 33). The membrane-bound isoforms of AC are widely distributed and differentially regulated by protein kinases and calcium (6, 9).

The increase of cGMP was not unexpected since we showed that guanylyl cyclase coupled receptors were expressed in acinar cells. Furthermore, increased cGMP in response to ANF has been reported in the pancreas (10), as well as in response to CNP in other tissues (29). The increase in cGMP supports NPR-A and/or NPR-B receptor activation, although these receptors were not involved in CNP-evoked amylase secretion as previously discussed. The stimulation of cGMP showed a biphasic response possibly related to receptor desensitization induced by increased PKC activity, mediated by NPR-C, since this kinase mediates the heterologous desensitization of natriuretic peptide receptors coupled to guanylyl cyclase (21). Secretagogues that increase intracellular calcium levels also increase cGMP content, but current evidence supports the idea that this messenger is not directly involved in the triggering of the exocytotic process in the exocrine pancreas but in the modulation of calcium entry across the cell membrane (19, 20). Whether CNP-induced cGMP is involved in the regulation of amylase release in the exocrine pancreas remains to be elucidated.

We reported that ANF also stimulates pancreatic fluid and protein secretion in vivo through the activation of NPR-C receptors coupled to the PLC pathway but without affecting AC signaling unless stimulated by secretin or VIP (27, 24). These findings indicate that, although both natriuretic peptides modulate exocrine pancreatic secretion, the underlying mechanisms are different.

In the present study we showed that the three natriuretic peptide receptors were expressed in the rat exocrine pancreas. Although guanylyl cyclase-coupled receptor expression was reported in human pancreas, the presence of NPR-C receptors has not been demonstrated in this tissue (11). Therefore whether the effect of CNP on amylase release through NPR-C activation occurs also in humans is presently unknown.

In conclusion, the present study shows that the three natriuretic peptide receptors were expressed in the exocrine pancreas and that CNP stimulated amylase release through the activation of NPR-C receptors coupled to the activation of PLC and downstream effectors. The attenuation of amylase release at higher concentrations of the peptide was likely related to the reduction of cAMP observed at the same concentrations. The augmentation in cGMP indicates activation of NPR-A/NPR-B receptors probably involved in calcium influx once exocytosis is triggered. Present findings further support the idea that the natriuretic peptides are not only relevant peptides in the control of the cardiovascular and renal activity, but they may also be part of the large family of peptides involved in the regulation of the digestive function.

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