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ORIGINAL ARTICLE

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Effect of uncoupling NO/cGMP pathways on carbachol- and CCK-stimulated Ca^{2+} entry and amylase secretion from the rat pancreas

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Abstract Nitric oxide (NO) production reportedly regulates guanosine 3′,5′-cyclic monophosphate (cGMP) formation and Ca^{2+} influx in pancreatic acini. We have investigated the functional roles of the NO/cGMP messenger system in rat pancreatic acini. In dispersed acini, the levels of amylase secretion, cytosolic $[Ca^{2+}]$($[Ca^{2+}]_i$), NO synthase, and cGMP were measured. The NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 0.01–100 μM) had no effect on amylase secretion induced by various concentrations of carbachol, cholecystokinin octapeptide (CCK-8) or the high affinity CCK agonist, JMV-180. Similarly, L-NAME up to 100 μM did not affect the changes in Ca^{2+} spiking evoked by these secretagogues; nor was Ca^{2+} entry, refilling or oscillation altered by L-NAME. Sub- and supramaximal concentrations of these secretagogues did not change NO synthase activities compared with basal levels. While sodium nitroprusside (SNP), a NO donor, caused a 9.4-fold increase in cGMP levels compared with basal levels, carbachol, CCK-8 and JMV-180 had no effect. In addition, the guanylate cyclase inhibitor LY 83583 (10 nM to 10 μM) altered neither amylase secretion nor Ca^{2+} signaling induced by these secretagogues. These findings indicate that the stimulatory action of carbachol or CCK-8 is not mediated by NO or cGMP. To investigate whether cGMP stimulates pancreatic secretion we showed that both SNP and a cell-permeant cGMP analog at 0.1–1 mM stimulated amylase secretion and Ca^{2+} transients to a level equal to 10–15% and 13–24%, respectively, of those observed with maximal concentrations of secretagogues. The guanylate cyclase activator guanylin (1–10 μM), which increased cGMP levels 2.4-fold compared with basal levels, elicited a small amount of amylase secretion and a small Ca^{2+} transient. In conclusion, exogenous NO is capable of increasing endogenous cGMP, which results in a modest increase in the $[Ca^{2+}]_i$ transient and pancreatic amylase secretion. However, the NO/cGMP system does not appear to be involved significantly in the mediation of Ca^{2+} signaling and amylase secretion stimulated by carbachol and CCK-8.

Key words Nitric oxide · cGMP · Ca^{2+} entry · Pancreas

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Introduction

Guanosine 3′,5′-cyclic monophosphate (cGMP) is a well-characterized intracellular signal transducer in the mediation of stimulus–secretion and excitation–contraction coupling [7]. Nitric oxide (NO) is an endogenous molecule of significant biological importance in mammalian cells [17]. It acts by stimulating soluble guanylate cyclase and the subsequent cGMP pathway [17].

In pancreatic acinar cells, interactions between NO, cGMP and pancreatic enzyme secretion have been recognized previously, but direct roles for NO and cGMP in mediating the secretory process are controversial [4, 11–13]. Haymovits and Scheele [12] have reported that, in guinea pig pancreatic acini, several secretagogues, such as carbamylcholine chloride (carbachol) and cholecystokinin (CCK), which are Ca^{2+}-mobilizing agents [38], increase cellular cGMP levels and high concentrations of cGMP analogs cause amylase secretion at a level approximately 30% of that observed with a maximal concentration of carbachol. This suggests that cGMP may be an intracellular mediator of pancreatic amylase secretion. In contrast, Gunther and Jamieson have observed that, in guinea pig pancreatic acini, sodium nitroprusside (SNP) elevates levels of cGMP 60-fold over basal, but it does not trigger amylase secretion [11]. Similarly, Gardner and Rottman [4] have demonstrated that, in guinea pig pancreatic acini, several Ca^{2+}-mobilizing secretagogues increase Ca^{2+} efflux, cGMP (18-fold increase) and amylase secretion, whereas SNP and hydroxylamine, which increase cGMP 45- to 72-fold over basal, respectively, fail to increase Ca^{2+} efflux or amylase secretion [4]. In rat pancreatic acinar cells,

phosphodiesterase inhibitors, which increase cGMP levels and elevate cGMP content induced by carbachol, do not alter amylase secretion under control conditions and during stimulation by carbachol [13]. These observations [4, 11, 13] suggest that cGMP does not play a major role in the stimulus–secretion coupling of pancreatic acinar cells.

In vivo studies of the dog pancreas have shown that SNP enhances pancreatic amylase secretion and elicits a twofold increase in cGMP content [14]. However, the increase in amylase secretion may be secondary to neuronal stimulation and increased blood flow, both of which may enhance canine pancreatic enzyme secretion [15]. This possibility is supported further by immunohistochemical data showing that, in rats, the NO synthase (NOS) system is present in neurons and the endothelium but not in pancreatic acinar cells [32].

Recently, Pandol and Schoeffield-Payne have demonstrated that, in guinea pig pancreatic acini, the guanylate cyclase inhibitor LY 83583 [19] reduces carbachol-stimulated cGMP formation, Ca^{2+} influx and Ca^{2+} refilling and that the inhibition of Ca^{2+} influx by LY 83583 is reversed by N^2,2′-*O*-dibutyryl cGMP (dbcGMP) and 8-Br-cGMP [23]. On the other hand, SNP increases both cGMP levels and the rate of Ca^{2+} efflux. These data therefore suggest that the carbachol-induced increase in cGMP is necessary and sufficient to mediate the effects of carbachol on the plasma membrane Ca^{2+}-entry mechanism. However, in a subsequent paper, the same authors have reported that when cells are stimulated with a Ca^{2+} ionophore cGMP once again increases [22]. In this instance LY 83583 augments the ionophore-induced Ca^{2+} signal and SNP inhibits the sustained Ca^{2+} entry induced by either the Ca^{2+} ionophore or carbachol [22]. More recently, the same group of investigators have reported that, in both guinea pig and rat pancreatic acini, inhibitors of the NOS, N^G-monomethyl-L-arginine (L-NMMA) and N^G-nitro-L-arginine (L-NAME), abolish the carbachol-induced cGMP rise and that this inhibition is reversed by the addition of excess L-arginine, suggesting a role for NO in cGMP formation [9]. In addition, carbachol increases [^{3}H]arginine conversion to [^{3}H]citrulline, indicating that the pancreatic acini possess the NOS system. L-NMMA suppresses the stimulation of Mn^{2+} quenching by carbachol in fura-2-loaded acini, suggesting that the NOS system coupled to Ca^{2+}-mobilizing agents may regulate plasma membrane Ca^{2+} influx via the mediation of guanylate cyclase/cGMP pathways [9]. Muallem and his associates have reported similarly that, in rat pancreatic acini, both L-NAME and LY 83583 inhibit Ca^{2+} entry induced by either carbachol or the endoplasmic reticulum Ca^{2+} pump inhibitor, thapsigargin [41]. Such inhibition is reversed by the administration of exogenous NO donors and dbcGMP. It was also demonstrated that carbachol, thapsigargin and NO_2 increased cGMP levels (8- to 12-fold increase over basal). It is interesting to note that this study showed cGMP to have a dual action on Ca^{2+} entry: a tenfold increase in cGMP over basal activated Ca^{2+} entry, whereas an 80-fold increase in cGMP over basal inhibited Ca^{2+} entry. In streptolysin-O-permeabilized acini, supramaximal Ca^{2+} loading (2.5 μM) only modestly increased NOS activity, whereas the depletion of Ca^{2+} from intracellular stores and submaximal [Ca^{2+}] (0.3 μM) markedly increased NOS activity [41]. It was therefore proposed that the stimulation of Ca^{2+} entry involves the release of Ca^{2+} from intracellular stores, activating a cellular pool of NOS which in turn generates cGMP, with this modulating Ca^{2+} influx through the plasma membrane.

The sustained Ca^{2+} entry across the plasma membrane during agonist-evoked stimulation is essential to maintain amylase secretion from pancreatic acinar cells [37]. The mechanism(s) responsible for this process still remain(s) unclear. If the NO/cGMP system is indeed capable of mediating Ca^{2+} influx activated by carbachol or CCK, it will play a critical role in mediating pancreatic enzyme secretion. Given the conflicting data in the literature, we performed in vitro studies to examine the role of NO/cGMP in mediating Ca^{2+} signaling and amylase secretion in rat pancreatic acinar cells.

Materials and methods

Materials

Chemicals were purchased from the following sources: carbachol, CCK octapeptide [Tyr(SO_3H)^{27}]-fragment 26–33 amide (CCK-8), L-arginine, SNP, 3-isobutyl-1-methylxanthine (IBMX) and $NiCl_2$ from Sigma (St. Louis, Mo., USA); CCK 27–32(Nle^{28,31})-2-phenylethylester, sulfated, protected (JMV-180) from Research Plus (Bayonne, N.J., USA); L-NAME, 6-anilinoquinoline-5-8-quinone (LY 83583), 8-Br-cGMP sodium salt, dbcGMP sodium salt and guanylin from Biomol (Plymouth Meeting, Pa., USA); 1-(2–5′-carboxyoxazol-2′-yl)-6-aminobenzofuran-5-oxy-(2′-amino-5′-methylphenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (fura-2) acetoxymethyl ester (AM) from Calbiochem (San Diego, Calif., USA). L-Arginine, IBMX, L-NAME, 8-Br-cGMP, dbcGMP, carbachol, $NiCl_2$ and guanylin were dissolved in distilled water stock solutions of 0.1–1 M. LY 83583 (100 mM stock solution), fura-2/AM (1 mM stock solution) and JMV-180 (1 mM stock solution) were dissolved in 100% dimethyl sulfoxide, the final concentration being 0.01%, 0.2% and less than 0.01%, respectively. CCK-8 (1 mM stock solution) was dissolved in distilled water containing 0.01 N NaOH and 0.2% bovine serum albumin.

Methods

Isolation of pancreatic acini

Isolated rat pancreatic acini were prepared by collagenase digestion of pancreas obtained from male Sprague-Dawley rats [39]. Acini were suspended in a physiological salt solution (PSS) containing 0.1% bovine serum albumin, 0.1 mg/ml soybean trypsin inhibitor and (in mM): 137 NaCl, 4.7 KCl, 0.56 $MgCl_2$, 1.28 $CaCl_2$, 1.0 NaH_2PO_4, 10.0 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), Eagles' minimum essential amino acid neutralized with NaOH, and 2.0 L-glutamine. The PSS was adjusted to pH 7.35 and equilibrated with 100% O_2. In experiments evaluating the NOS activity (see Fig. 3) and in the amylase secretion study using L-arginine (see Fig. 4), the Eagles' minimum essential amino acid was removed from the PSS (pH 7.35 adjusted by NaOH) since it contained 0.6 mM L-arginine. In the medium used for Ca^{2+}-free studies (Figs. 5, 8, 9 and 10), the Ca^{2+}-free solution ($-[Ca^{2+}]_o$) was identical to PSS except that $CaCl_2$ was replaced by

with 1 mM ethyleneglycol bis(β-aminoethylether)*N*,*N*,*N*′,*N*′-tetra-acetic acid (EGTA).

Measurement of amylase secretion and $[Ca^{2+}]_i$

For the measurement of amylase secretion, acini were preincubated for 30 min in 40 ml PSS, washed twice (50 *g* times for 3 min), and resuspended in 40 ml fresh PSS. Aliquots (2 ml) were then distributed into each flask and incubated with reagents for 60 min at 37°C. The incubation was terminated by centrifugation (10,000 rpm) for 40 s at 4°C in a microfuge (1 ml x 2 times in 20 groups). Amylase released into the supernatant and that remaining in the pellet in each tube was assayed using Procion yellow starch (Polysciences, Inc. Pa., USA) as a substrate. Amylase secretion was expressed as a percentage of the total content in each sample.

$[Ca^{2+}]_i$ in individual pancreatic acini was measured as described previously [36]. In brief, acini were incubated with 2 μM fura-2/AM at 37°C in 10 ml PSS for 30 min. All experiments utilized a dual-excitation-wavelength (340/380 nm emitted at 505 nm) modular fluorometer system (SPEX Fluorolog 2) coupled to a Nikon Diaphot inverted microscope (x40). Isolated acini placed on a zero cover glass and mounted in the closed chamber were continuously superfused from an eight-chambered reservoir (1 ml/min). The fluorescence ratio was converted to $[Ca^{2+}]_i$ using in vitro calibration with an external standard (Calcium Kit I, Molecular Probes/Eugene, Ore., USA) and 25 μM fura-2 potassium salt [8].

Measurement of NOS activity

NOS activity was assessed by measuring [^{3}H]arginine conversion to [^{3}H]citrulline. [^{3}H]Arginine (3 μl, 3 μCi/111 kBq, Dupont NEN products, Boston, Mass., USA) was added to 1-ml aliquots of the acinar cell suspension in 1.5-ml Eppendorf plastic tubes (Madison, Wis., USA)(2×10^6 cells/PSS without Eagles' minimum essential amino acids) and incubated for 5 min at 37°C. The cell suspension was stimulated immediately with secretagogues for 0–10 min at 37°C and centrifuged at 10,000 rpm at 4°C for 20 s. The supernatant was removed and the resultant pellet immediately frozen in liquid nitrogen. The pellet was stored at –70°C overnight, resuspended in 0.5 ml of 5 mM L-arginine in 4 mM ethylenediaminetetraacetic acid, (EDTA) buffer (pH 7.8), homogenized 10 times using a glass homogenizer, and transferred to a new Eppendorf tube. This process was repeated twice (final volume 1 ml). Each suspension was centrifuged at 10,000 rpm for 1 min at 4°C and the supernatant collected in a glass tube. Then, 1 ml of 20% trichloroacetic acid (TCA) was added and the mixture sonicated in a water bath for 5 min at 4°C. Each suspension was again centrifuged at 2,000 rpm for 5 min at 4°C and 1 ml of the supernatant transferred to a new glass tube (13×100 mm). Then, 2 ml of H_2O-saturated ether was added to each tube and the tube vortexed for 30 s and the upper phase was discarded. This process was repeated 3 times. The aqueous fraction (1 ml) was evaporated at 55°C under a N_2 stream for 1 min. The remaining lower phase (0.5 ml) was transferred to a new glass tube (30×100 mm) and 2 ml of 20 mM HEPES solution (pH 6.0) added. Cation-exchange columns were prepared by adding 2.6 ml of Bio-Rad analytical grade cation exchange resin (AG 50W×8 resin, Bio-Rad, Hecules, Calif., USA) to each column and prewashing twice with 2 ml of 20 mM HEPES. Samples (2.5 ml) were poured onto respective columns and collected in scintillation vials. Each original tube was washed with 2 ml of 20 mM HEPES, vortexed and poured over the same column into a new scintillation vial. This process was repeated once more. After adding 10 ml of Ecolite (ICN, Costa Mesa, Calif., USA), the radioactivity was counted using a liquid scintillation counter. The background (no samples) was subtracted from each sample counting rate (counts per minute) and values from the sample and two rinses which had been passed through the one column were summed. Data are expressed as the percentage of basal/100 μg protein. The protein content of each sample before TCA treatment was measured using a Bio-Rad protein assay system. Chromatographic procedures performed in this study resulted in the retention of 98.1% of [^{3}H]arginine in the resin.

Measurement of cellular cyclic GMP

Cellular cGMP was measured using a radioimmunoassay using the [^{3}H]cGMP assay system (Amersham, Arlington Heights, Ill., USA). Aliquots (0.25 ml) of the acinar cell suspension (2×10^6 cells in PSS) were incubated with secretagogues at 37°C for 0–20 min. Incubation was stopped by adding 0.5 ml of chilled 99.9% ethanol and immediately centrifuged at 10,000 rpm for 20 s at 4°C. The supernatant (0.75 ml) was stocked at 4°C and the resultant pellet dissolved in 0.5 ml chilled 99.9% ethanol:distilled water (2:1). The suspension was sonicated for 30 s (200 W, Braun-Sonic L), vortexed for 30 s and allowed to settle for 10 min at 4°C. The sonicates were centrifuged at 10,000 rpm for 10 min and the resultant supernatant (0.5 ml) combined with the first supernatant (1.25 ml total volume). Each supernatant was evaporated at 55°C under a N_2 stream for 20 min to remove the ethanol fraction completely. Each sample was stored at –70°C overnight and redissolved in 0.3 ml of 0.05 M tris(hydroxymethyl)aminomethane (Tris)-EDTA buffer (pH 7.5). A 0.1-ml aliquot of each suspension (20–100 μg protein/cell extract) was incubated with an antiserum specific for cGMP (0.05 ml) and 0.008 μCi/0.295 kBq of [^{3}H]cGMP (0.05 ml), both of which were solubilized in 0.05 M Tris-EDTA buffer. The final volume per incubation was 0.2 ml. Incubation was performed for 90 min at 7°C with gentle shaking and stopped by adding 1 ml of chilled and 60%-saturated $(NH_4)_2SO_4$ solution. Bound and free labeled cGMP were separated by centrifugation at 10,000 rpm for 3 min at 4°C. After completely removing the supernatant, the resultant pellet was resolubilized with 1.1 ml of distilled water in each tube. All procedures were performed using 1.5-ml Eppendorf plastic tubes. A 1-ml aliquot of the suspension was transferred into a scintillation vial and mixed with 15 ml of Cytoscinti (ICN). The radioactivity in each vial was counted using a liquid scintillation counter. The protein content of the cell extract in the same fraction utilized for cGMP assay was measured using a Bio-Rad protein assay system. cGMP contents were expressed in picomoles per milligram protein per cell extract (cytosolic protein). Basal cGMP values were approximately 10 pmol/mg protein per cell extract. This means that almost 1 pmol of cGMP was present in the total amount of protein (mg).

Results

The NOS inhibitor L-NAME has no effect on amylase secretion and $[Ca^{2+}]_i$ response induced by either carbachol, CCK-8 or JMV-180

As shown in Fig. 1a, pretreatment of dispersed pancreatic acini with a supramaximal concentration of the competitive NOS inhibitor L-NAME [26] (100 μM) for 10 min and further treatment for 60 min with L-NAME plus secretagogues did not change the amylase secretory pattern induced by either carbachol [0.1–100 μM, $P>0.8$, $F = 8.66\times10^{-3}$, data (n) = 20, SD within two groups (± L-NAME) = 12.01 by one-way analysis of variance (ANOVA)], CCK-8 (10–100 pM, $P>0.8$, $F = 6.51\times10^{-3}$, n = 12, SD = 16.02) or the high-affinity CCK receptor agonist JMV-180 (10–100 nM, $P>0.8$, $F = 6.38\times10^{-3}$, n = 12, SD = 13.02). Figure 1b shows the effects of different concentrations of L-NAME on basal, unstimulated amylase secretion (US) as well as on amylase secretion induced by carbachol (1 μM), CCK-8 (10 pM) or JMV-

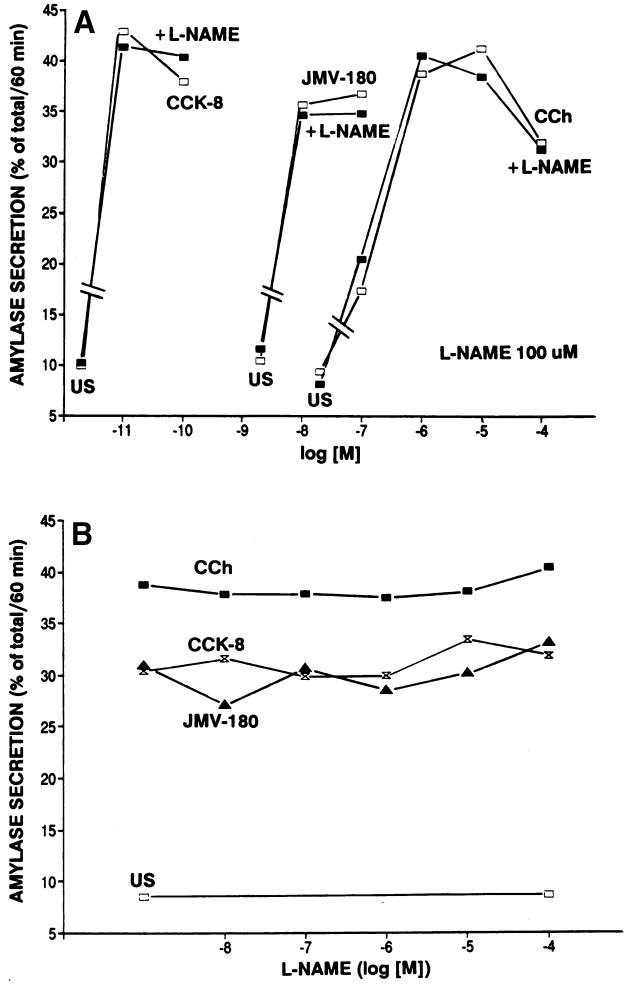

Fig. 1A, B Effects of N^G-nitro-L-arginine methylester (*L-NAME*), a nitric oxide synthase (*NOS*) inhibitor, on amylase secretion in dispersed rat pancreatic acinar cells induced by carbachol (*CCh*), cholecystokinin octapeptide (*CCK-8*) and the high affinity CCK analog, *JMV-180*. **a** L-NAME (100 μM) had no effect on CCh- (0.1–100 μM), CCK-8- (10–100 pM) and JMV-180- (10–100 nM) induced amylase secretion. **b** CCh- (1 μM), CCK-8- (10 pM) and JMV-180- (10 nM) induced amylase secretion was not altered by various doses of L-NAME (0.01–100 μM). Data are means of duplicate determinations (*US* Unstimulated cells i.e., basal)

180 (10 nM). All responses were unaffected by various concentrations of L-NAME (0.01–100 μM).

Next, we determined the effects of L-NAME on Ca^{2+} signaling induced by various secretagogues in fura-2-loaded individual pancreatic acini. Addition of 100 μM L-NAME to the superfusion medium did not change the carbachol-induced (10 μM) sustained $[Ca^{2+}]_i$ plateau, which is dependent on the presence of extracellular Ca^{2+} ($[Ca^{2+}]_0$)[37]. On the other hand, the Ca^{2+} channel blocker $NiCl_2$ (2 mM) completely abolished the sustained $[Ca^{2+}]_i$ plateau induced by carbachol (Fig. 2a). As shown in Fig. 2b and reported previously [34], after a 10-min washout the second Ca^{2+} spike in response to carbachol was completely restored. This requires refilling of the intracellular Ca^{2+} stores from extracellular sources [24]. Application of 100 μM L-NAME during the refilling phase did not alter the second Ca^{2+} response induced by carbachol, indicating that Ca^{2+} entry across the plasma membrane is unaffected by L-NAME. Similarly, neither the sustained $[Ca^{2+}]_i$ plateau induced by CCK-8 (10 nM) nor the Ca^{2+} oscillations evoked by JMV-180 (100 nM) were altered by the administration of L-NAME (Fig. 2c,d).

Carbachol, CCK-8 and JMV-180 have no significant effect on NOS activity

Since the NOS inhibitor L-NAME had no effect on either amylase secretion or Ca^{2+} signaling in response to various secretagogues, we next established whether the NOS system is present and functioning in pancreatic acini. NOS activity was measured by [^{3}H]arginine conversion to [^{3}H]citrulline in an Eagles' essential amino-acid-free PSS. Figure 3a shows the time course of NOS activity during carbachol-evoked stimulation. No significant increases in [^{3}H]citrulline were observed at any time in response to stimulation by carbachol (300 μM). In addition, L-NAME (100 μM) (10-min preincubation and co-incubation with carbachol) did not affect NOS activity during carbachol-evoked stimulation. Figure 3b shows [^{3}H]citrulline formation in response to various concentrations of carbachol (1–300 μM), CCK-8 (10–100 pM) and JMV-180 (10–100 nM) 5 min after cell stimulation. There were no significant increases over basal following stimulation by the various secretagogues. L-NAME (100 μM, 10-min preincubation and 5-min co-incubation with carbachol) had no significant effect on the action of various concentrations of carbachol [$P = 0.53$, $F = 0.40$, $n = 29$, SD within two groups (± L-NAME) = 30.18 by ANOVA]. The 1.5-fold increase in NOS activities observed at some concentrations was not statistically significant because of the large standard deviations.

L-Arginine has no effect in inducing amylase secretion

To examine further whether the NOS system functions in pancreatic acini, we studied amylase secretion using L-arginine. Intact acini were incubated with L-arginine (10 μM to 3 mM) in the PSS, which did not include the Eagles' essential amino acid. L-Arginine up to 3 mM did not cause significant increases in amylase secretion (Fig. 4). Note that the PSS utilized for other experiments (except those shown in Figs. 3 and 4) contained 0.6 mM L-arginine and this concentration of L-arginine did not significantly increase basal amylase secretion during 60 min of stimulation. Furthermore, L-NAME (100 μM, 10-min preincubation and 60-min co-incubation with L-arginine) did not alter the secretory pattern of amylase induced by L-arginine. Note that carbachol, CCK-8, and JMV-180 caused amylase secretion reaching 25–45% of the total content. These results suggest that the NOS system is either not present or not functioning in rat pancreatic acini.

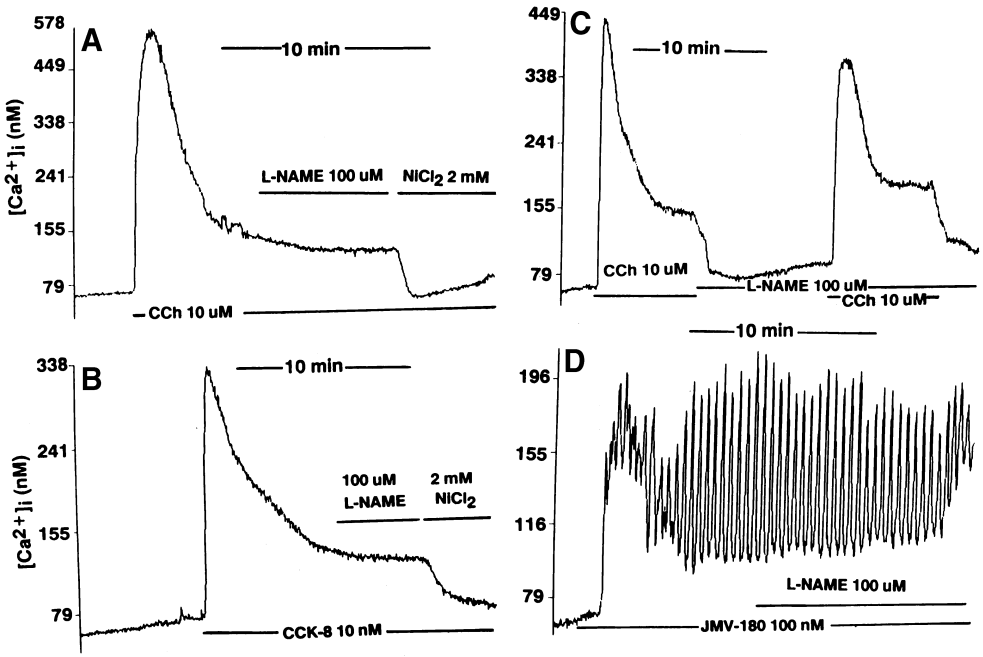

Fig. 2A–D Effects of L-NAME on Ca^{2+} signaling expressed as intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) evoked by CCh, CCK-8 and JMV-180 in individual pancreatic acini. Neither Ca^{2+} entry (**a** and **c**), Ca^{2+} refilling (**b**) nor Ca^{2+} oscillations (**d**) were altered by L-NAME (100 μM). Data are representative of ten separate experiments

SNP, but not carbachol, CCK-8 or JMV-180, increases cellular cGMP levels

Several reports have suggested that the guanylate cyclase/cGMP system is present in pancreatic acini but does not mediate stimulus–secretion coupling [4, 11, 13]. To address this question, cellular cGMP levels were measured using a [^{3}H]cGMP radioimmunoassay kit. As shown in Fig. 5a, addition of SNP (100 μM) resulted in a biphasic increase in cellular cGMP content with peak increases of 4- and 4.4-fold at 2 min and 10 min respectively. In contrast, carbachol (300 μM) caused no increase in cellular cGMP levels over basal in both the presence or absence of the phosphodiesterase inhibitor, IBMX (100 μM). Although small increases in cGMP levels (2.0- to 2.5-fold over that at zero time) in response to the supramaximal concentration of carbachol were observed early after cell stimulation (0.5–1 min), these changes were not significant. cGMP (9.1 ± 1.5 pmol/mg protein per cell extract, n = 8) after 10 min of carbachol-evoked (300 μM) stimulation without IBMX was almost similar to that in unstimulated cells (9.0 ± 0.8 pmol/mg protein per cell extract, n = 4). Figure 5b depicts the effects of different concentrations of SNP and carbachol on cGMP content 10 min after stimulation. SNP caused a linear concentration/response increase in cGMP content with a concentration for half-maximal effect (EC_{50}) of 3 μM. SNP (1 mM) caused a 9.4-fold increase in cGMP level over basal (10.0 ± 1.3 pmol/mg protein per cell extract, n = 6). The stimulatory action of SNP appeared to require the presence of $[Ca^{2+}]_o$ for its full activation (Fig. 5c). Similar $[Ca^{2+}]_o$-dependent, but small, increases in cGMP have been observed recently in rat pancreatic acini in response to the Ca^{2+} adenosine triphosphatase (ATPase) inhibitor thapsigargin (2 μM) [6]. Preincubation of acini with 10 μM LY 83583 (10 min) reduced cGMP content stimulated by 1 mM SNP [50.7 ± 7.7 (+LY 83583) vs 93.7 ± 22.3 (–LY 83583) pmol/mg protein per cell extract at 10 min, P<0.05 by two-tailed unpaired t-tests, n = 6], indicating that the LY 83583 used in this study, and which did not inhibit secretagogue-stimulated amylase secretion or Ca^{2+} responses (see Figs. 6 and 7), was biologically active. On the other hand, carbachol (0.1–300 μM) had no effect on cellular cGMP content in either the presence or absence of IBMX after 10 min of cell stimulation (Fig. 5b). Note that 0.1 mM IBMX caused an 1.5-fold increase in basal cGMP levels, suggesting that the cGMP/phosphodiesterase system is present in rat pancreatic acini.

Neither CCK-8 (100 pM) nor JMV-180 (100 nM) altered cGMP levels from basal levels, which were 11.0 ± 2.4 pmol/mg protein per cell extract in this series of experiments. At 10 min following stimulation by CCK-8 and JMV-180 the cGMP levels were 9.8 ± 3.4 and 14.6 ± 7.0 pmol/mg protein per cell extract, respectively (each n = 5). Similarly, L-arginine (1 mM) during 10 min of cell stimulation did not increase cGMP levels (8.0 ± 3.3 pmol/mg protein per cell extract, n = 6) over basal.

The guanylate cyclase inhibitor LY 83583 has no effect on amylase secretion and $[Ca^{2+}]_i$ response induced by carbachol, CCK-8 and JMV-180

To examine further whether or not the guanylate cyclase/cGMP system is coupled to amylase secretion and $[Ca^{2+}]_i$ signaling in response to carbachol, CCK-8 and JMV-180, we examined the effects of LY 83583, a guanylate cyclase inhibitor [19]. As shown in Fig. 6a,

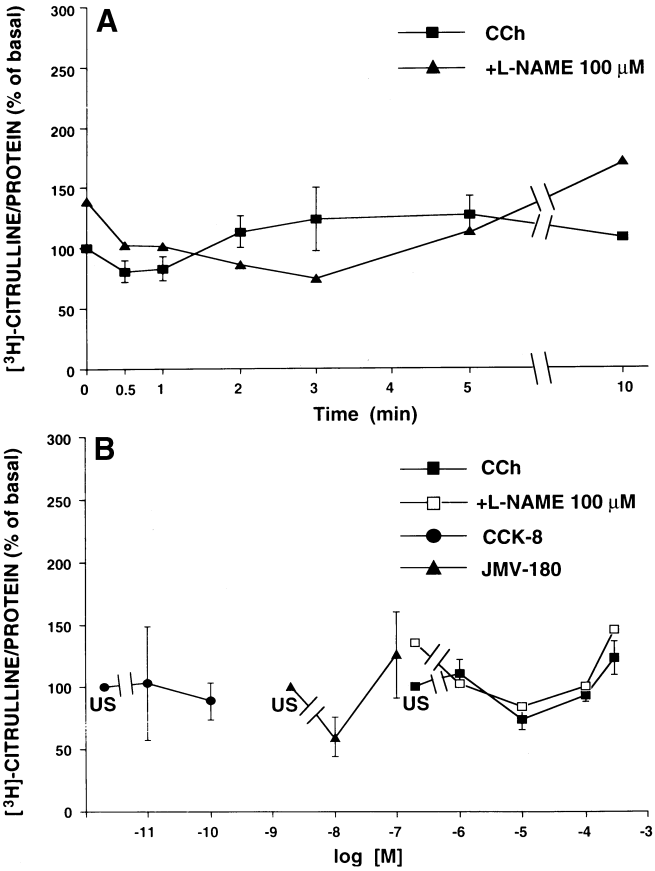

Fig. 3A Time course of NOS activity (expressed as rate of [^{3}H]citrulline formation) stimulated by 300 μM CCh in the presence or absence of 100 μM L-NAME. Data are mean ± SEM from four separate experiments. **B** Concentration/response curves for stimulation of NOS by CCh, CCK-8 and JMV-180 for 5 min. Data are mean ± SEM from four separate experiments. Acini were pretreated for 10 min with 100 μM L-NAME and further co-incubated with secretagogues. The value 100% corresponds to the zero-time or basal [^{3}H]citrulline levels. The medium used was an Eagles' essential amino-acid-free physiological salt solution (PSS)

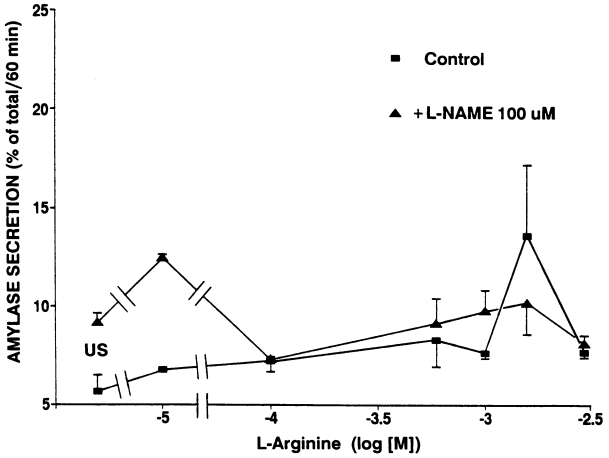

Fig. 4 Amylase secretion induced by L-arginine (0.01–3 mM) in the absence (*control*) or presence of L-NAME (100 μM). Data are mean ± SEM from three separate experiments (n = 3–7). The medium used was an Eagles' essential amino-acid-free PSS

10 μM LY 83583 (10-min preincubation and 60 min co-incubation with secretagogues) did not significantly inhibit amylase secretion induced by various concentrations of carbachol [0.1–100 μM, P = 0.76, $F = 9.35\times10^{-2}$, n = 20, SD within two groups (± LY 83583) = 5.05 by ANOVA], CCK-8 (10 pM to 10 nM, P = 0.25, F = 1.39, n = 24, SD = 5.21) and JMV-180 (1–1000 nM, P>0.8, $F = 2.54\times10^{-2}$, n = 20, SD = 12.36). Figure 6b depicts the effects of different concentrations of LY 83583 on the basal and stimulated amylase secretion induced by carbachol (10 μM), CCK-8 (10 pM) or JMV-180 (100 nM). No response was altered significantly by any concentration of LY 83583 (0.01–10 μM).

Next, we examined the effects of LY 83583 on Ca^{2+} signaling induced by the various secretagogues in fura-2-loaded individual acini. Application of 10 μM LY 83583 caused no significant inhibition of the carbachol-induced (10 μM) sustained $[Ca^{2+}]_i$ plateau , whereas the Ca^{2+} channel blocker $NiCl_2$ (2 mM) completely abolished this plateau (Fig. 7a). LY 83583, present for 10 min during the refilling phase, did not affect the second response elicited by carbachol (Fig. 7b). Similarly, neither the sustained $[Ca^{2+}]_i$ plateau induced by CCK-8 (10 nM) nor the Ca^{2+} oscillations evoked by JMV-180 (100 nM) were altered by LY 83583 (Fig. 7c, d). These data suggest that although the pancreatic acini possess the guanylate cyclase/cGMP system which is activated by exogenous NO donors it does not appear to be coupled to the signal transduction pathways utilized by carbachol, CCK-8 and JMV-180.

SNP and cell-permeant cGMP analogs cause a modest increase in amylase secretion

To investigate the biological functions of cellular cGMP, the effects of SNP and cell-permeant cGMP analogs on pancreatic amylase secretion were examined. As shown in Fig. 8a, SNP (1–100 μM) did not increase amylase secretion over basal. Only at 1 mM did SNP cause a 1.5-fold increase in amylase secretion over basal. This small increase in secretion elicited by SNP was dependent on the presence of $[Ca^{2+}]_o$. Similarly, the cell-permeant cGMP analog 8-Br-cGMP caused a 1.2-fold to 1.5-fold increase in amylase secretion only when supramaximal concentrations (0.1–1 mM) were used (Fig. 8b). Another cGMP analog, dbcGMP, also caused a modest increase in amylase secretion (1.25-fold over basal) only when a supramaximal concentration (1 mM) was used.

SNP and cell-permeant cGMP analogs cause small Ca^{2+} transient(s)

We next examined the actions of SNP and cell-permeant cGMP analogs on $[Ca^{2+}]_i$ signaling. SNP (0.1 mM) induced a Ca^{2+} transient in 18 out of 20 individual cells.

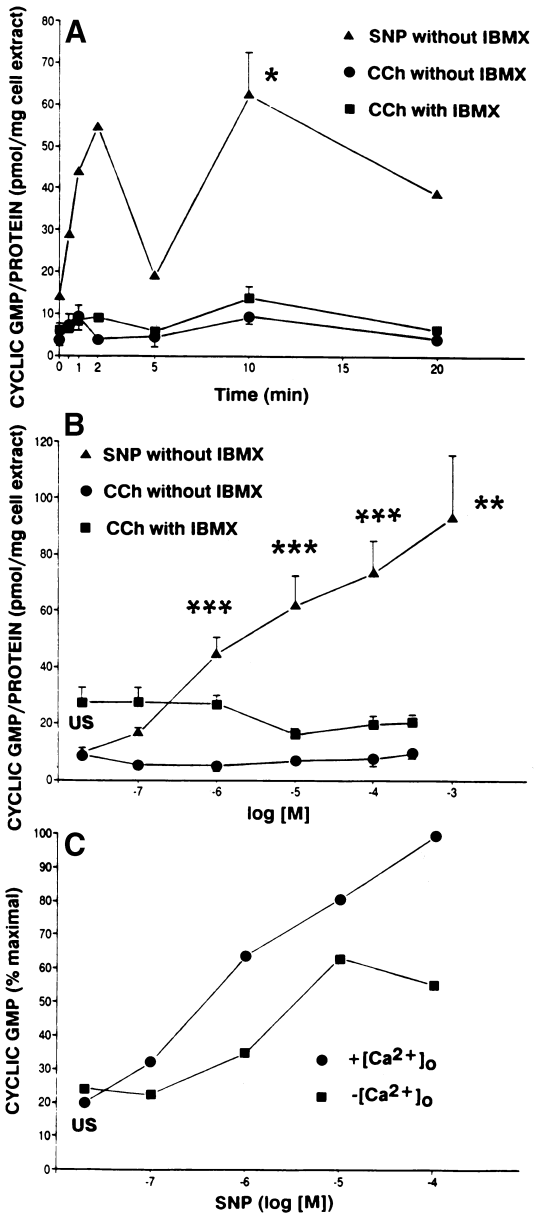

Fig. 5A–C Cellular guanosine 3′,5′-cyclic monophosphate (*cGMP*) levels stimulated by sodium nitroprusside (*SNP*) and CCh (300 μM). **A** SNP (100 μM) caused a biphasic increase in cellular cGMP content even in the absence of the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (*IBMX*, 100 μM). Data are mean ± SEM from two or three separate experiments (n = 2–4), $^{*}P<0.05$ vs zero time (two-tailed unpaired t-tests). **B** Concentration/response curves for effect of SNP(0.1 μM to 1 mM) and CCh (0.1–300 μM, with or without 100 μM IBMX) on cGMP production at 10 min. Data are mean ± SEM from two or three separate experiments (n = 4–12). $^{**}P<0.01$ and $^{***}P<0.001$ vs US (two-tailed unpaired t-tests). **C** Concentration/response curves for effect of SNP (0.1–100 μM) on cGMP production in the presence (+$[Ca^{2+}]_o$, *solid circles*) or absence (–$[Ca^{2+}]_o$, *solid squares*) of extracellular Ca^{2+} at 10 min. The 100% corresponds to cGMP levels measured when 100 μM SNP was used in the presence of extracellular Ca^{2+}. Each *point* is the mean of duplicate determination, P = 0.12, F = 2.64, n = 20, SD within two groups (± $[Ca^{2+}]_o$) = 13.95 by ANOVA

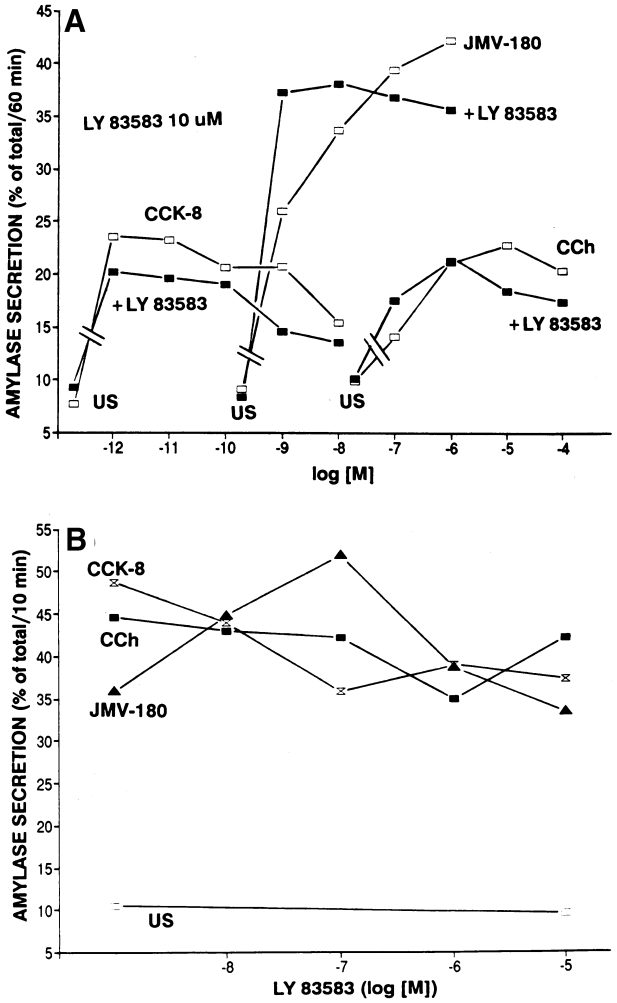

Fig. 6A, B Effects of the guanylate cyclase inhibitor LY 83583 on amylase secretion induced by CCh, CCK-8 and JMV-180. **A** LY 83583 (10 μM) had no effect on amylase secretion induced by various concentrations of CCh, CCK-8 and JMV-180. **B** Various concentrations of LY 83583 had no effect on basal (*US*) and stimulated amylase secretion induced by CCh (1 μM), CCK-8 (10 pM) and JMV-180 (10 nM). Each *point* is the mean from duplicate determinations

Basal $[Ca^{2+}]_i$ was 60 ± 9 nM. The $\Delta[Ca^{2+}]_i$ increase (peak–basal from each single cell) induced by SNP was 68 ± 13 nM (n = 20), a value 24% of that induced by 10 μM carbachol (289 ± 37 nM in 20 out of 20 cells). SNP (0.1 mM) failed to increase $[Ca^{2+}]_i$ in 11 out of 11 cells in the absence of Ca^{2+} from the medium (zero $CaCl_2$ plus 1 mM EGTA), indicating that the source of Ca^{2+} utilized for Ca^{2+} spiking stimulated by SNP is extracellular. In contrast to JMV-180, addition of SNP to the superfusion medium for 7–10 min did not cause Ca^{2+} oscillations but elicited only one transient. Lower concentrations of SNP (1–10 μM) that were sufficient to increase cGMP levels did not elicit Ca^{2+} spiking (see Fig. 5b), suggesting that 100 μM SNP is the threshold for evoking the Ca^{2+} response. 8-Br-cGMP at 0.1 mM caused a small and transient increase in $[Ca^{2+}]_i$ in 9 out

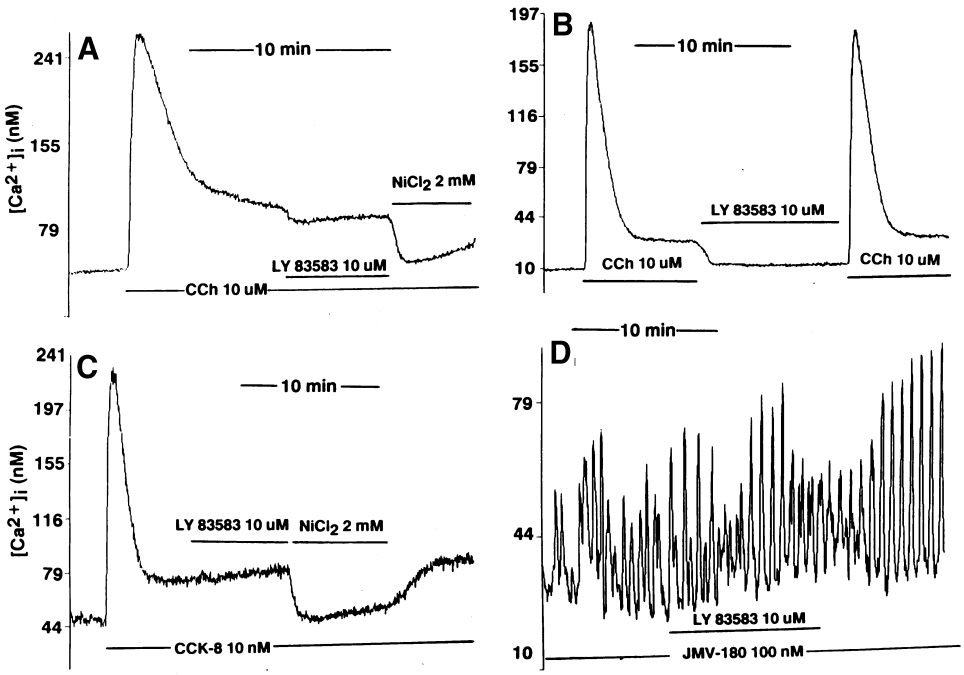

Fig. 7A–D Effects of LY 83583 on Ca^{2+} signaling induced by CCh, CCK-8 and JMV-180 in individual pancreatic acini. Neither Ca^{2+} entry (**A** and **C**), Ca^{2+} refilling (**B**), nor Ca^{2+} oscillations (**D**) induced by CCh, CCK-8 and JMV-180 were altered by LY 83583 (10 μM). Data are representative of eight separate experiments

of 15 cells (Fig. 9b). The $\Delta[Ca^{2+}]_i$ increase induced by 8-Br-cGMP was 26 ± 9 nM (n = 15), 13% of that induced by 10 μM carbachol in the same single cell (15 out of 15 cells). In contrast to SNP, the absence of extracellular Ca^{2+} did not abolish the $[Ca^{2+}]_i$ response induced by 8-Br-cGMP in 11 out of 18 responding cells: the $\Delta[Ca^{2+}]_i$ was 56 ± 15 nM (n = 18), 24% of that induced by 10 μM carbachol in 18 out of 18 cells (the $\Delta[Ca^{2+}]_i$ = 236 ± 24 nM in a Ca^{2+}-free medium, Fig. 9c). Similarly, dbcGMP (0.1 mM) caused a small transient increase in $[Ca^{2+}]_i$: the $\Delta[Ca^{2+}]_i$ increase was 33 nM in the presence of extracellular Ca^{2+} in two out of two cells (Fig. 9d). The mean $\Delta[Ca^{2+}]_i$ (peak–basal) value in response to secretagogues is calculated from all cells examined (responding and nonresponding). High concentrations of SNP and cGMP analogs were thus required to cause a small increase in amylase secretion and $[Ca^{2+}]_i$. The effects of SNP and cell-permeant cGMP analogs on Ca^{2+} signaling modes were, however, not always the same. This reason for this will be described later.

A guanylate cyclase activator, guanylin, causes modest amylase secretion and Ca^{2+} transient(s), and increases cellular cGMP levels

Current studies have suggested the existence of soluble guanylate cyclase, activated by SNP but not coupled to carbachol- or CCK-stimulated Ca^{2+} signal transduction or exocytosis in rat pancreatic acini. To investigate further whether pancreatic acini separately possess the transmembrane guanylate cyclase system (isoform C), we examined the action of the transmembrane guanylate cyclase activating peptide, guanylin (NH_2-Pro-Asn-Thr-Cys-Glu-Ile-Cys-Ala-Tyr-Ala-Ala-Cys-Thr-Gly-Cys-OH/cyclic) [1, 30, 31] on pancreatic secretion. Similar to SNP and cGMP analogs, only at a supramaximal concentration (10 μM) did guanylin evoke a small increase in amylase secretion in an extracellular Ca^{2+}-dependent manner (Fig. 10a). This increase was 39% of the maximal increase observed with 1 μM carbachol. Note that the accumulative amylase secretion in response to carbachol was completely dependent on the presence of extracellular Ca^{2+} [P = 0.0007, F = 13.75, n = 40, SD within two groups (± $[Ca^{2+}]_0$) = 8.23 by ANOVA, Fig. 10a]. This is consistent with our previous results showing that carbachol only elicits transient amylase secretion from superfused acini in the absence of extracellular Ca^{2+} [37].

In the absence extracellular Ca^{2+}, carbachol (10 μM) evoked a $[Ca^{2+}]_i$ transient without a sustained $[Ca^{2+}]_i$ plateau (Fig. 10b). Similarly, in the absence of extracellular Ca^{2+}, guanylin (1 μM) evoked a small $[Ca^{2+}]_i$ transient which was 39% of that observed with 10 μM carbachol in the same cell (n = 3), indicating that the source utilized for Ca^{2+} spiking evoked by guanylin and cGMP analogs is intracellular. On the other hand, the small sustained $[Ca^{2+}]_i$ plateau elicited by guanylin or cGMP analogs seems to originate from the extracellular compartment. In the presence of extracellular Ca^{2+}, guanylin (1 μM) elicited a small, sustained $[Ca^{2+}]_i$ plateau (86 nM increase over basal) (Fig. 10b). At lower concentrations, guanylin (0.01–0.1 μM) did not elicit a $[Ca^{2+}]_i$ increase. Since only the supramaximal concentration of guanylin (10 μM) caused amylase secretion, we measured cGMP level at this concentration. As expected, guanylin caused a biphasic increase in cellular cGMP levels with significant peak increases occurring at 1 min and 20 min

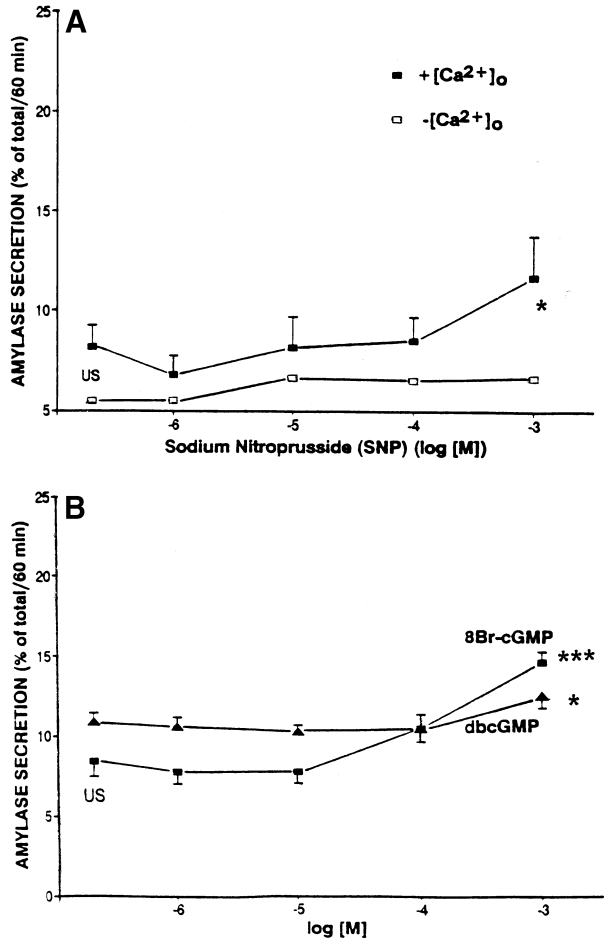

Fig. 8A, B Effects of SNP and cGMP analogs on amylase secretion. **A** Concentration/response curves for the effect of SNP on amylase secretion in the presence (*closed squares*) or absence (*open squares*) of extracellular Ca^{2+}. **B** Concentration/response curves for 8-Br-cGMP and dibutyryl cGMP (*dbcGMP*) on amylase secretion. Each *panel* shows means ± SEM from three or four separate experiments (n = 4–9) $^{*}P<0.05$, $^{***}P<0.001$ compared with US (two-tailed, unpaired t-tests)

(Fig. 10c). Results suggest that, in addition to the soluble guanylate cyclase system activated by exogenous NO, the transmembrane guanylate cyclase system is present in rat pancreatic acini. However, it does not appear to be coupled to carbachol- or CCK-stimulated Ca^{2+} signal transduction or pancreatic amylase secretion.

Discussion

In this study, we have demonstrated that, in the rat pancreatic acini, 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 pathways utilized by carbachol and CCK-8. These data are consistent with previous findings that increasing cellular cGMP is not necessarily coupled to amylase secretion during receptor activation in guinea pig and rat pancreatic acini [4, 11, 13]. Furthermore, immunohistochemical studies with measurements of reduced nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase activity, a marker for NOS, have demonstrated that the NOS system is present in the rat pancreatic neurons (nerve cell bodies) and endothelium, but not in pancreatic acinar cells [32]. In addition, Ca^{2+}/calmodulin-dependent type I NOS has been found in pancreatic β cells, but not in pancreatic acinar cells [29].

In the first series of studies (Figs. 1 and 2), we used L-NAME as a NOS inhibitor because of the lower concentration required for 50% inhibition (IC_{50}, 0.54 μM) for NOS compared with L-NMMA (IC_{50} = 9.5 μM) in vitro [26]. Our studies showed that neither amylase secretion nor Ca^{2+} spiking (entry, refilling and oscillations) induced by carbachol, CCK-8 and JMV-180 was altered by different concentrations of L-NAME, suggesting that the NOS system does not function in rat pancreatic acini. Pretreatment time (10 min) and concentrations (up to 100 μM) of L-NAME utilized in this study seem to be sufficient to inhibit guanylate cyclase activities in intact cells [9]. These observations differ from those of two recent studies which have reported that L-NAME and L-NMMA inhibit NOS, cGMP production, $^{45}Ca^{2+}$ uptake and Mn^{2+} influx in rat and guinea pig pancreatic acini [9, 41]. Gukovskaya and Pandol demonstrated that, in guinea pig and rat pancreatic acini, L-NMMA (10–1000 μM) dose-dependently inhibit NOS activity (1.5-fold over basal), cGMP content (sevenfold over basal), $^{45}Ca^{2+}$ uptake (1.5-fold increase over basal) and Mn^{2+} entry evoked by 100 μM carbachol [9]. In contrast, the same group also reported that, in guinea pig pancreatic acini, the NO donor SNP (30 μM) decreases the sustained $[Ca^{2+}]_i$ plateau evoked by either carbachol (300 μM) or the Ca^{2+} ionophore Br-A23187 (10 μM) and that the guanylate cyclase inhibitor LY 83583 (0.1–3 μM) augments the $[Ca^{2+}]_i$ increase induced by Br-A23187 (4 μM)[22]. Although the reason for this discrepancy is unknown, these observations suggest that NO produced in pancreatic acini may regulate the soluble guanylate cyclase system which produces cGMP. This may mediate either the Ca^{2+} influx, Ca^{2+} efflux or a combination of both. Similarly, Muallem and his associates have reported that, in individual rat pancreatic acinar cells, a supraphysiological concentration of L-NAME (2 mM) inhibits carbachol-evoked (100 μM) Mn^{2+} quenching by 74% and that induced by thapsigargin (2 μM) by 78% [41]. These studies were performed using a fura-2 fluorescence quenching rate at the isosbestic point (360 nm) and the 360/380 ratio. It should be noted that technical difficulties may be encountered when Mn^{2+} quenching studies are used as an index of Ca^{2+} influx across the plasma membrane. In some cases, Mn^{2+} quenches fura-2 fluorescence even in the resting state [35]. Furthermore, the Mn^{2+} quenching rate is different in each individual cell, so that both careful quantitative and qualitative analyses

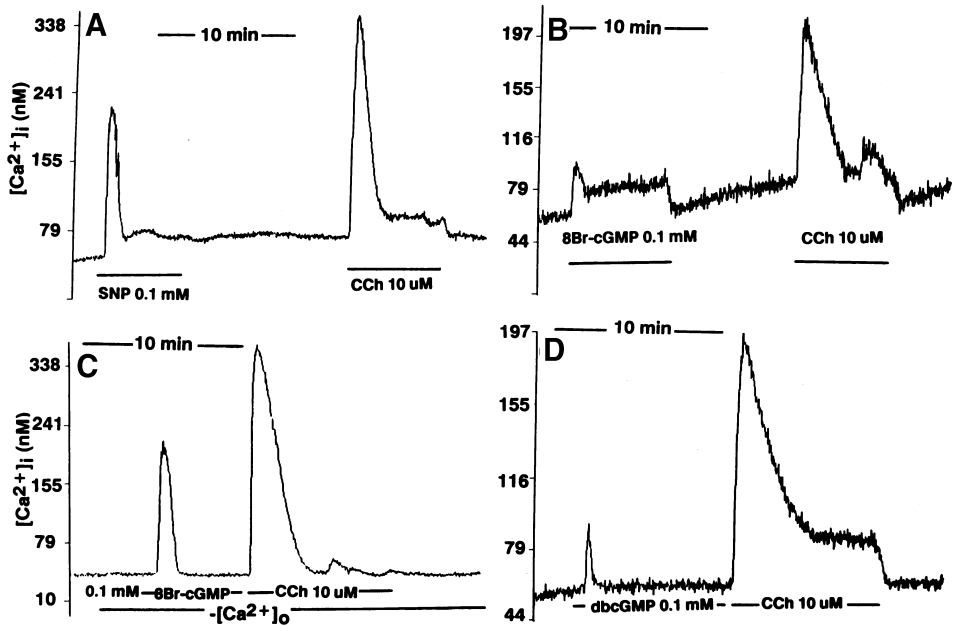

Fig. 9A–D Ca^{2+} signaling (as shown by $[Ca^{2+}]_i$) induced by SNP, CCh and cGMP analogs in individual pancreatic acini. **A** SNP (0.1 mM) effect, representative of 18 out of 20 cells. **B** Responses to 8-Br-cGMP (0.1 mM), representative of 9 out of 15 cells. The experiments shown in **A** and **B** were carried out in the presence of extracellular Ca^{2+}. **C** Responses to 0.1 mM 8-Br-cGMP (11 out of 18 cells) in the absence of extracellular Ca^{2+}. **D** Responses to 0.1 mM dbcGMP (2 out of 2 cells) in the presence of extracellular Ca^{2+}

are needed. On the other hand, Putney and his associates [6] have reported recently that 2 mM L-NAME had a very small inhibitory effect on capacitative Ca^{2+} entry evoked by 2 μM thapsigargin.

We have reported previously that the sustained $[Ca^{2+}]_i$ plateau (50 nM over basal) is critical for maintaining amylase secretion in rat pancreatic acini [35, 37]. This study also showed that amylase secretion induced by carbachol was completely dependent on the presence of extracellular Ca^{2+} (1.28 mM). Thus sustained Ca^{2+} entry from the extracellular space is a necessary intermediate for a long-term cellular response [35]. However, the precise mechanism by which Ca^{2+} enters the cells via unidentified Ca^{2+} channels remains to be determined for all types of non-excitable cells. Recent studies have suggested that the NO/cGMP system may play a role in regulating Ca^{2+} entry and sustaining amylase secretion [9, 41]. Our study, however, clearly showed that L-NAME had no effect on amylase secretion and Ca^{2+} mobilization during stimulation by CCK and carbachol. Furthermore, NOS activity was not enhanced significantly during stimulation evoked by carbachol, CCK-8 or JMV-180. In addition, we also showed that L-arginine (10 μM to 3 mM) elicited little or no increase in amylase secretion. It appears, therefore, that the NOS enzyme responsible for converting L-arginine to L-citrulline is either not present or not functioning in the pancreatic acini. Alternatively, even if the NOS system is present [9, 10, 40, 41], it is not linked to the signal transduction pathways utilized by carbachol and CCK. Furthermore, our results do not confirm the finding of Wrenn et al. that showed that, in rat pancreatic acini, L-NAME inhibits carbachol-stimulated (1 μM) amylase secretion with an approximate IC_{50} of 2.2 μM [40].

Several previous reports have shown that the NO donors and cGMP analogs, which markedly increase cellular cGMP, fail to elicit amylase secretion from pancreatic acinar cells [4, 11, 13]. This suggests that the NO/cGMP system may not be involved in the secretory process in mammalian pancreatic acini. Another possibility, as pointed out by Muallem and his associates, is that cGMP has a dual action: a modest increase of cGMP (up to tenfold over basal, elicited by secretagogues) activates Ca^{2+} entry, whereas large increases of cGMP (up to 80-fold over basal, as caused by high concentrations of SNP) inhibit Ca^{2+} entry [41]. Thus large increases in cellular cGMP induced by NO donors or cGMP analogs may fail to stimulate enzyme secretion. However, this phenomenon may not be applicable to stimulus–secretion coupling events, since we observed no significant increase in $[Ca^{2+}]_i$ or amylase secretion with submaximal SNP concentrations (0.1–10 μM), which were accompanied by a modest increase in cGMP (see Figs. 5b and 8a).

Similar to previous reports [4, 6, 9–13, 20, 33, 40, 41], this study clearly indicates that the soluble guanylate cyclase/cGMP system, which can be activated by exogenous NO donors, is present in rat pancreatic acini. The stimulation of cellular cGMP by NO was partially dependent on the presence of extracellular Ca^{2+} (1.28 mM). Previous studies have reported that Ca^{2+}-mobilizing secretagogues cause a 4- to 20-fold increase in cellular cGMP level over basal [4, 9, 11–13, 20, 23, 33, 40, 41]. Recently, with the availability of a selective cGMP antiserum, measurements of cGMP levels have become more reliable. Using a radioimmunoassay system, we demonstrated that carbachol, CCK-8 and JMV-180, at concentrations which are sufficient to elicit an increase in amylase secretion and $[Ca^{2+}]_i$, did not increase cellular cGMP levels significantly over different time courses. Only 2.0- to 2.5-fold increases in cGMP in response to a supramaximal carbachol concentration (300 μM) were observed shortly after cell stimulation, in

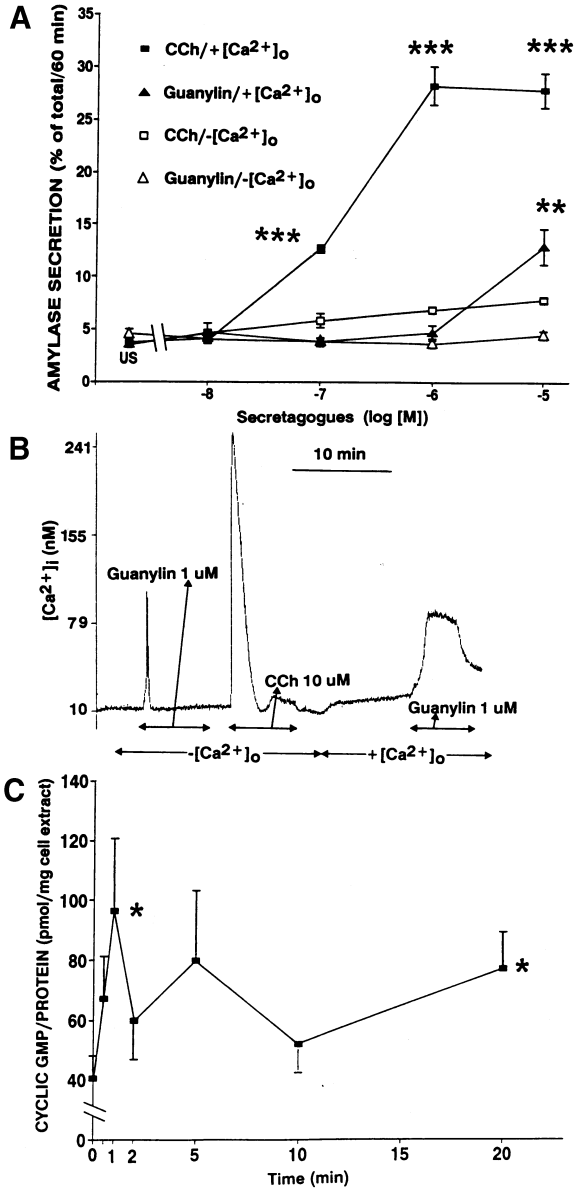

Fig. 10 Effects of the guanylate cyclase activator, guanylin on **A** amylase secretion, **B** Ca^{2+} signaling and **C** cellular cGMP levels. **A, B** Responses are compared with those to CCh and studied in the absence or presence of extracellular Ca^{2+}. **A** Mean ± SEM from two separate experiments (n = 4, $^{**}P$<0.01, $^{***}P$<0.001 compared with US, two-tailed unpaired t-tests). **B** $[Ca^{2+}]_i$ response to guanylin (1 μM) and CCh (10 μM) in the presence or absence of extracellular Ca^{2+} (n = 3). **C** Time course of cGMP production in response to 10 μM guanylin. Means ± SEM from two separate experiments (n = 6–8), $^{*}P$<0.05 compared with the zero time, two-tailed, unpaired t-tests

which levels were similar to those after stimulation by thapsigargin (2 μM) in rat pancreatic acini [6]. These data suggest that the soluble guanylate cyclase/cGMP system is not coupled significantly to the signal transduction pathways utilized by these secretagogues. This view is further supported by the observations that various concentrations of the guanylate cyclase inhibitor LY 83583 did not significantly affect amylase secretion induced by different concentrations of carbachol, CCK-8 and JMV-180. In addition, LY 83583 (10 μM) did not affect either sustained Ca^{2+} entry, refilling or oscillations induced by these secretagogues. Pretreatment time (10 min) and concentrations (1–10 μM) of LY 83583 utilized in this study are sufficient to inhibit the soluble guanylate cyclase activity in intact cells [19, 23].

To demonstrate further that the NO/cGMP system is not linked significantly to amylase secretion, we showed that SNP and cGMP analogs caused little (at 100–1000 μM) or no (at 1–10 μM) increase in amylase secretion. This result again indicates that NO/cGMP pathways do not correlate with carbachol- or CCK-stimulated Ca^{2+} entry or pancreatic amylase secretion, as initially proposed by Gunther and Jamieson [11]. The present observations are similar to those reported by Gardner and his associates who have shown that, in rat pancreatic acini, supramaximal concentrations of SNP (10 mM) and 8-Br-cGMP (1 mM) do not alter basal or stimulated amylase secretion evoked by carbachol (3 μM) or CCK-8 (0.1 nM) [16]. The small increase in amylase secretion induced by either SNP or cGMP analogs at 100–1000 μM may be triggered by the small and transient increase in $[Ca^{2+}]_i$. Furthermore, this study showed that guanylin (the transmembrane guanylate-cyclase-activating peptide [1, 30, 31]), at a supramaximal concentration (10 μM), evoked only a small increase in amylase secretion, although it markedly increased cellular cGMP levels. This suggests that, in addition to the soluble guanylate cyclase, rat pancreatic acini possess the transmembrane guanylate cyclase system which, however, is not coupled to carbachol- or CCK-stimulated Ca^{2+} signal transduction or pancreatic exocytosis. Of interest, Ca^{2+} transient(s) induced by SNP were dependent on extracellular Ca^{2+}, whereas the Ca^{2+} transient(s) elicited by guanylin were derived from intracellular stores. This difference may be due to differences in the action of SNP, which activates the soluble guanylate cyclase and guanylin, which acts on the cytoskeleton-associated particular guanylate cyclase [1, 17]. As a consequence, the differing localization of intracellular cGMP may result in different actions of Ca^{2+} signaling. Cell-permeant cGMP analogs may thus cause both intracellular Ca^{2+} release and extracellular Ca^{2+} entry.

cGMP elicits different patterns of Ca^{2+} signaling in different cell types. A typical pattern, in which cGMP enhances the influx of Ca^{2+} from the extracellular space (through Na^+/Ca^{2+} channels), is seen in photoreceptors in the dark [42]. These intracellularly activated, cGMP-gated Ca^{2+} channels are voltage sensitive and belong to the superfamily II. A similar phenomenon is observed in cAMP-gated Ca^{2+} channels in olfactory neurons [2]. In macrophages and sea urchins, cGMP may release Ca^{2+} from intracellular stores [3, 25]. This may be regulated by cyclic adenosine diphosphate (ADP) ribose which is synthesized by cGMP [3]. On the other hand, in smooth muscle cells and other cell types, the NO/cGMP cascade may mediate relaxation by decreasing $[Ca^{2+}]_i$ [5, 21]. cGMP is more effective in inhibiting the Ca^{2+} influx in-

duced by ADP in platelets [18], but has no effect on mediating Ca^{2+} influx in endothelial cells [28]. Thus, cGMP may interact with $[Ca^{2+}]_i$ differentially in different cells types. We found that, in rat pancreatic acini, activators of both the soluble and transmembrane guanylate cyclase systems cause small increases in $[Ca^{2+}]_i$ in 60–100% of responding cells, but neither system is coupled to receptor-operated Ca^{2+} entry.

As has been proposed by Sutherland and his associates [27], a minimum of four criteria should be met if NOS/NO and the guanylate cyclase/cGMP systems are to serve as second messengers in mediating secretion of the pancreatic acini: (1) the agonists should be able to increase the production of NO and cGMP; (2) increased NO and cGMP should precede the secretory response to the agonists; (3) addition of NO-generating agents and cGMP analogs should reproduce the response to the agonists; (4) addition of antagonists of NOS and guanylate cyclase should reduce or abolish the secretory response to the agonists. Our results indicate that none of these criteria could be met, thus indicating that the NO/cGMP system is not linked to the Ca^{2+} signal transduction pathways utilized by carbachol and CCK-8. Similar findings have been reported recently for rat pancreatic acini: the rise in cGMP levels appears to depend on, rather than cause, the increase in $[Ca^{2+}]_i$ with agonist-evoked stimulation [6]. Recent studies using inhibitors of NO production have suggested that endogenous NO may be involved in the control of pancreatic secretion [15]. However, these studies did not identify the source(s) of NO responsible for pancreatic stimulation. It is conceivable that neural release of NO in the pancreas may increase the soluble guanylate cyclase activity and subsequent cellular cGMP production. This may result in a modest increase in $[Ca^{2+}]_i$ and amylase secretion, independent of carbachol- or CCK-coupled signal transduction pathways. Furthermore, neural release of NO may also increase pancreatic blood flow, which may contribute further to stimulation of pancreatic enzyme secretion [15, 32].

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