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FULL PAPER

Nitric oxide stimulates IP_3 production via a cGMP/PKG-dependent pathway in rat pancreatic acinar cells

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Abstract

In an attempt to explore the functioning of nitric oxide (NO) in pancreatic exocrine cells, we have recently obtained several lines of circumstantial evidence indicating that one of molecular targets of NO is phospholipase C (PLC), the activation of which leads to an increase in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) via inositol 1, 4, 5-trisphosphate, IP_3. However, whether IP_3 is actually produced by NO has not yet been substantiated. The present study was therefore designed to directly measure the intracellular IP_3 concentration ($[IP_3]_i$) for better understanding of the underlying mechanisms with the help of pharmacological tools. $[IP_3]_i$ was measured using a fluorescence polarization technique (HitHunter™). We obtained the following results: 1) varying concentrations of an NO donor, sodium nitroprusside (SNP), elevated $[IP_3]_i$, 2) this elevation was completely inhibited in the presence of the soluble guanylyl cyclase (sGC) inhibitor, 1H-[1, 2, 4] oxadiazolo [4, 3-a] quinoxalin-1-one (ODQ), 3) varying concentrations of the cGMP analogue, 8-Br-cGMP, also increased $[IP_3]_i$, 4) the cGMP analogue-induced IP_3 production was abolished by pretreatment with either a PLC inhibitor, U73122, or a G-protein inhibitor, GP2A, and 5) KT5823, a potent and highly selective inhibitor of cGMP-dependent protein kinase G (PKG), also abolished the IP_3 production induced by 8-Br-cGMP. These results suggest that the NO-induced $[Ca^{2+}]_i$ increase is triggered by an increase in $[IP_3]_i$ located downstream from intracellular cGMP elevation. In this intracellular pathway, each sGC, cGMP-dependent PKG, G-protein and PLC were suggested to be involved. The present work provides new insights into the intracellular signaling accelerated by NO. NO triggers a $[Ca^{2+}]_i$ increase via cGMP and IP_3 in pancreatic acinar cells.

Key words: Ca^{2+}, cGMP, IP_3, nitric oxide, pancreatic acini

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Introduction

Nitric oxide (NO) plays critical roles in various biological systems. This bioactive gas is formed from an amino acid, L-arginine, via a reaction catalyzed by NO synthases (NOSs) that are either constitutive or inducible[20] in the presence of molecular oxygen. Physiologically diverse effects exerted by this gaseous radical are regarded to be intermediated largely by soluble guanylyl cyclase (sGC), which is considered to be a receptor for NO. Cyclic GMP produced by catalytic action of sGC activates cGMP-dependent protein kinase G (PKG) to phosphorylate an array of proteins[22].

G-protein-mediated intracellular Ca^{2+} signaling has long been depicted as a key event that initiates exocrine secretion in pancreatic acini. The signaling commences from binding of G-protein-coupled receptors with a variety of agonists followed by activation of phospholipase C (PLC), which promotes elevation of the intracellular IP_3 concentration ($[IP_3]_i$) in such a way that this phosphoinositide metabolite triggers the release of Ca^{2+} from intracellular stores[35]. Lines of evidence have demonstrated that NO could induce Ca^{2+} release in several cell types[3,26] either via PKG-coupled activation of ADP-ribosyl cyclase, resulting in increased synthesis of a potent Ca^{2+} mobilizing agent, cyclic ADP-ribose[3,36], or via direct nitrosylation of regulatory thiol groups of ryanodine receptors[30]. Thus, in some cells, Ca^{2+} and NO appear to work synergistically in the regulation of cellular homeostasis. However, some controversial findings have also been reported regarding the effect of cGMP on the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) depending on the type of cell. Elevation of $[Ca^{2+}]_i$ has been described in hepatocytes[28] and sea urchin eggs[9,11] and suppression of $[Ca^{2+}]_i$ was noted in cardiac myocytes[19], cerebellar neurons[8] and vascular smooth muscle cells[5]. The idea that phosphorylation of the IP_3 receptor by PKG potentiates IP_3-dependent Ca^{2+} release was proposed for hepatocytes[28], whereas it was hypothesized that phosphorylation of ADP ribosyl cyclase stimulates the synthesis of cyclic ADP-ribose, which potentiates Ca^{2+}-induced Ca^{2+} release from ryanodine-sensitive stores in sea urchin eggs[9]. For G-protein-coupled receptors, negative modulation by cGMP has been shown. Decreased generation of the two major second messengers, IP_3 and diacylglycerol, and blunting of Ca^{2+} release from intracellular stores were demonstrated[18]. In these receptors, the site of modulation was presumed to be at the G-protein/PLC interface[26].

NO is well known to have a functional inhibitory role on IP_3 production in anterior pituitary cells[33], megakaryocytes[31] and ciliary and iris sphincter smooth muscle cells[6]. In the pancreatic exocrine gland, where it is known that NOSs exist, however, the functional role of NO in the machinery of exocrine secretion, especially in terms of $[IP_3]_i$, is unclear. Recent studies in our laboratory suggested that NO could mobilize Ca^{2+} from IP_3-sensitive stores via cGMP and/or a PLC pathway in pancreatic acinar cells[21] but its detailed cascade is not known. Accordingly, the present work was designed to further explore the NO-related signaling pathway by especially focusing on actual changes in $[IP_3]_i$ and to obtain insights into the overall signaling pathway accelerated by NO mediating $[Ca^{2+}]_i$ dynamics in pancreatic acinar cells.

Materials and Methods

Chemicals: Chromatographically purified collagenase (CLSPA) was purchased from Worthington Biochemical (Lakewood, NJ, USA). U-73122, dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), Dulbecco's phosphate-buffered saline containing neither $CaCl_2$ nor $MgCl_2$ (PBS), soybean trypsin inhibitor (type1-S) and the cGMP analogue, 8-Br-cGMP, were from Sigma (St. Louis, MO, USA). HEPES was from Dojindo (Kumamoto, Japan). Eagle's essential amino acid (MEM) without L-glutamine was

purchased from Invitrogen (Carlsbad, CA, USA). Sodium nitroprusside (SNP) and 1H-[1, 2, 4] oxadiazolo [4, 3-a] quinoxalin-1-one (ODQ) were obtained from Wako Pure Chemicals (Osaka, Japan). A G_q-protein antagonist peptide, GP2A[27], and a PKG inhibitor, KT5823, were purchased from Calbiochem (La Jolla, CA, USA).

Solutions: Normal Ringer's solution used for acinar isolation (Standard HEPES-buffered solution) contained (mM): NaCl, 138.0; KCl, 4.7; $CaCl_2$, 1.3; $MgCl_2$, 1.13; Na_2HPO_4, 1.0; D-glucose, 5.5; HEPES, 10.0 supplemented with MEM plus 2 mM L-glutamine, 1 mg/ml BSA and 0.1 mg/ml soybean trypsin inhibitor. The pH was adjusted to 7.4 with NaOH.

Isolation of pancreatic acini: All experiments conformed to the guidelines on the ethical use of animals set by the U.S. National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University. All efforts were made to minimize animal suffering and to reduce the number of animals used. Adult specific pathogen-free male Wistar rats weighing 200–250 g purchased from Clea Japan (Tokyo, Japan) were used throughout the experiments. The animals were fasted overnight with free access to water and maintained at 22°C in an air-controlled room with a 12:12-h light-dark cycle. The animals were anesthetized by CO_2 inhalation and euthanized by exsanguination. The pancreata were removed quickly and freed from fat and lymph nodes. The pancreatic acini were obtained by collagenase digestion according to a method reported previously[2]. Briefly, 5 ml of standard solution containing 60–75 U/ml collagenase was injected into the interstitium of the pancreatic tissue and digested in a conical flask at 37°C under vigorous shaking for a total of 60 min. After 30 min of digestion, old collagenase in the flask was replaced with 5 ml of new collagenase. Mechanical disruption of the tissue was performed by gentle suction through pipettes with decreasing orificial size. The acinar suspension was then filtrated through 150 μm nylon mesh, rinsed at least twice with PBS, pelleted (x60 g) and resuspended in a suitable amount of PBS. Acinar cell viability was virtually 100% when assessed by the trypan blue exclusion test.

Measurement of $[IP_3]_i$: The amount of IP_3 was measured using HitHunter™ IP_3 Fluorescence Polarization Assay Kits (DiscoveRx Tech, Fremont, CA, USA), a reliable and convenient methodology based on competitive binding between an IP_3 fluorescence tracer and unlabeled IP_3 from the cell lysates or standards. Free IP_3 competes at the IP_3 binding protein and allows the IP_3 tracer to rotate freely upon excitation with plane polarized light. The polarized signal is inversely proportional to the amount of the free unlabelled IP_3; thus, as the concentration of IP_3 is increased the polarization signal is decreased[25]. Black 384-well plates (Greiner Bio-One, Frickenhausen, Germany) were used for measurement of IP_3. Briefly, cells were treated with different agonists (SNP or 8-Br-cGMP) for designated periods in the presence or the absence of various inhibitors. The cellular reaction was terminated by placing cells on ice followed by addition of 0.2 N perchloric acid to lyse the cells. The plate was then shaken at 650 rpm for 5 min. The IP_3 tracer was subsequently added to each well and the IP_3 binding protein was finally added to the plate. The polarized fluorescence from the IP_3 tracer (fluorescein) was read using a Synergy™ 4 Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT) with a polarization mirror, and 485 nm excitation filter and 530 nm emission filter. The $[IP_3]_i$ was calculated from the IP_3 standard curve, which was prepared from 3-fold serial dilutions of IP_3 standards ranging from 1.33×10^{-6} to 6.7×10^{-11} M and fitted with a four-parameter Hill equation.

Statistical analysis: Data obtained from triplicate assays were collected from at least three independent experiments. The $[IP_3]_i$ was expressed as the percent of prestimulated basal $[IP_3]_i$ (nM) by setting the basal IP_3 concentration as 100% (baseline). The area under the curve (AUC) was also calculated for the whole time course (300 sec) to assess IP_3 production (% change) and represented as (AUC percent·sec). Data are presented as mean ± SE. The statistical analysis was performed using Student's *t*-test with a value of $P < 0.05$ being considered significant.

Results

SNP-induced IP_3 production

Pancreatic acini were stimulated with the NO donor, SNP, and IP_3 production was directly measured. As shown in Fig. 1a, SNP (50 μM) induced a marked increase in $[IP_3]_i$ and this increase appeared to be oscillatory with a frequency of approximately one spike per 2 min, which was equivalent to that of NO-induced Ca^{2+} oscillation[21]. Different concentrations of SNP from 20 μM to 1 mM also elevated $[IP_3]_i$ (Fig. 1b). All concentrations of SNP tested induced almost the same degree of elevation with some decrease at a higher concentration. This finding suggested that NO activated PLC as IP_3 was produced from PLC-mediated hydrolysis of PIP_2.

Involvement of sGC/cGMP in NO-induced IP_3 production

Generally, a guanylyl cyclase, especially sGC, is considered to be one of the most essential proteins activated by NO and a consequence of activation of this enzyme is an increase in the intracellular cGMP concentration. We therefore next tried to evaluate the possible involvement of sGC/cGMP in the NO-activated intracellular pathway from the viewpoint of IP_3 production. Two prominent inhibitors were applied. First, isolated acini were pretreated with ODQ, a potent and selective inhibitor of NO-sensitive guanylyl cyclase (GC). Pretreatment with ODQ at 100 μM for 30 min before and 5 min during SNP application almost completely inhibited the 50 μM SNP-induced $[IP_3]_i$ increase as shown in Fig. 2a. The AUC of IP_3 production was found to decrease by 97% in the presence of ODQ (Fig. 2b). This result implied a principal role of GC, mainly sGC, in the pathway of NO-induced IP_3 production. As the downstream product of sGC is cGMP, we next applied 8-Br-cGMP, a membrane-permeable and stable analogue of cGMP, and examined whether, as with the NO donor, IP_3 production could be induced to further

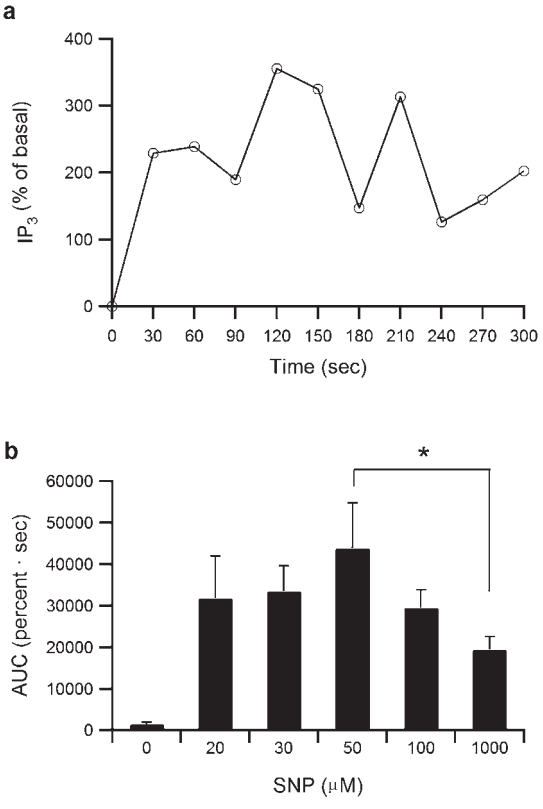

Fig. 1. SNP-induced IP_3 production. (a) Time course of IP_3 production induced by 50 μM SNP. Intracellular IP_3 was measured every 30 sec and normalized as % of basal value. (b) Isolated pancreatic acini were stimulated with different concentrations of SNP. The actual IP_3 concentrations in nM were measured every 30 sec for 5 min and converted to % of basal. Then the area under the curve (AUC) was obtained. Each column represents mean ± SE of AUC (n = 3–4). *P < 0.05 by Student's *t*-test.

substantiate the potential involvement of sGC. As expected, application of 8-Br-cGMP at varying concentrations ranging from 0.1 μM to 1 mM was also found to induce oscillatory $[IP_3]_i$ increases (Fig. 3a–d). The IP_3 level was increased by 1.7- to 2.4-fold over the basal level. These results indicated that NO stimulated IP_3 formation via the sGC/cGMP signaling pathway.

Stimulation of PLC by cGMP

A general understanding that IP_3 is formed by catalytic activity of PLC would raise the question of whether the cGMP-induced IP_3 production found in the foregoing experiments was mediated by PLC. To address this question, acini were pretreated with a PLC inhibitor, U73122, and cGMP-induced IP_3 production was measured. The $[IP_3]_i$ elevation induced by 0.1 μM 8-Br-cGMP was significantly inhibited (by 66%) by the pretreatment of acini with 2 μM U73122 for 30 min when compared with the untreated control value (Fig. 4a, b). This result indicated that cGMP was able to activate PLC and induce subsequent IP_3 production. The next question is by what mechanism cGMP activates PLC. We focused on G-protein, as PLC is activated by an α-subunit cleaved from trimeric G-protein. The acini were preincubated with a G_q-protein antagonist peptide (10 μM), GP2A, for 30 min before and 5 min during stimulation with 0.1 μM 8-Br-cGMP. As depicted in Fig. 4b, IP_3 production induced by 8-Br-cGMP was markedly diminished in the presence of GP2A, which was also obvious in the time course of 8-Br-cGMP-induced changes in $[IP_3]_i$ (Fig. 4a). The IP_3 production was found to decrease by 72% (Fig. 4b). From these results, we speculated that cGMP most likely stimulated IP_3 production, not completely but largely, via a well-characterized G-protein/PLC pathway.

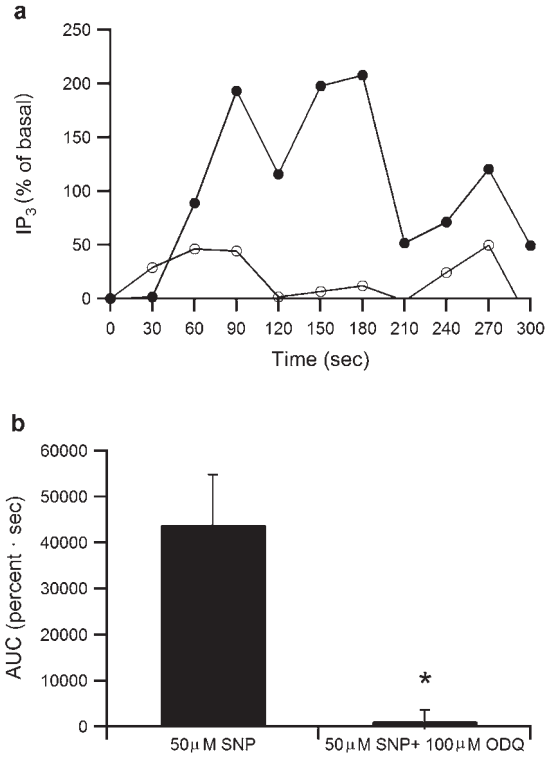

Fig. 2. Effect of ODQ on SNP-induced IP_3 production. (a) Time course of IP_3 production induced by 50 μM SNP in the presence (open circle) and absence (closed circle) of 100 μM ODQ. (b) AUC in the presence and absence of 100 μM OQD. Values are means ± SE (n = 3). *$P < 0.05$.

Effect of KT5823 on cGMP-induced IP_3 production

It is documented that the intracellular action of cGMP is primarily mediated by a cGMP-dependent PKG, but several types of cyclic nucleotide-activated ion channels are also reported to be involved[7,17]. Thus, whether PKG mediated cGMP-induced IP_3 production was examined by using a highly cell-permeable and selective inhibitor of PKG, KT5823[10,13]. Pretreatment with 10 μM KT5823 for 30 min significantly blocked cGMP-induced $[IP_3]_i$ elevation by 86% (Fig. 4b), indicating that the stimulatory effect of the cGMP analogue depended upon activation of PKG to elicit IP_3 production.

Discussion

NO-induced IP_3 production

NO is an essential intracellular messenger involved in a variety of crucial physiological events. Previous work from our group

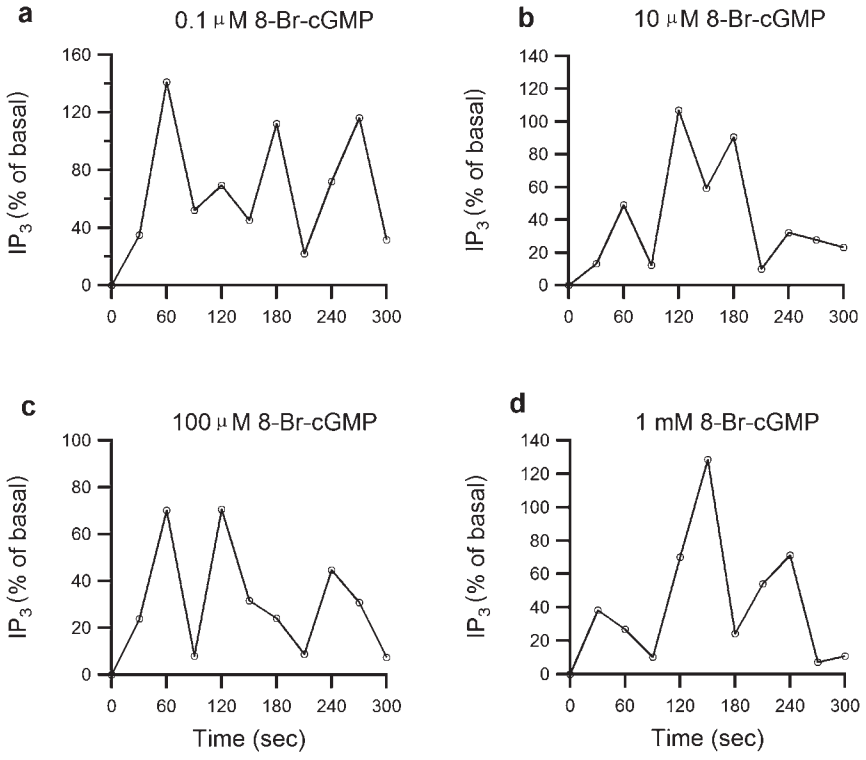

Fig. 3. 8-Br-cGMP-induced IP_3 production. Typical examples of time courses of IP_3 production induced by 0.1 (a), 10 (b), 100 μM (c) and 1 mM (d) 8-Br-cGMP. IP_3 concentration was measured every 30 sec for 5 min.

demonstrated a novel NO/PLC signaling pathway relaying NO-induced Ca^{2+} oscillation and activation of cGMP, PLC, IP_3 and resultant Ca^{2+} release from IP_3-sensitive stores in rat pancreatic acini[21]. Several reports have shown that NO, through PKG activation, modulates phosphoinositide metabolism[4,37], sensitivity of the IP_3 receptor[13,15] and PLC activation, which results in production of IP_3, triggering Ca^{2+} release from nonmitochondrial intracellular stores[10,14]. However, little is known about the mechanisms of the NO action on inositol phosphate metabolism in pancreatic acinar cells. In this study, we especially focused on actual IP_3 production by NO or cGMP in pancreatic acini to further substantiate our previous assumption.

A stimulatory effect by NO gas on IP_3 formation has been reported only in chromaffin cells[24]. On the other hand, an inhibitory action has been documented in anterior pituitary cells[33], megakaryocytes[31] and ciliary and iris sphincter smooth muscle cells[6]. For pancreatic exocrine cells, no reports are available in which the $[IP_3]_i$ was directly measured after NO/cGMP stimulation. In the current study, we attempted to actually measure the IP_3 level in isolated rat pancreatic acini in order to examine the effects of NO on IP_3 production and tried to better understand the underlying mechanism of NO-induced Ca^{2+} release. First, we found that an NO donor, SNP, induced oscillatory changes in $[IP_3]_i$ with a frequency of approximately one spike per two minutes, which appeared to be mostly identical to the frequency of NO-induced Ca^{2+} dynamics in pancreatic acinar cells[21]. This would indicate that NO-induced $[Ca^{2+}]_i$ oscillation occurred due to oscillatory changes in $[IP_3]_i$. In other words, this implies a strong correlation between IP_3 and Ca^{2+} in the NO effect. Thus, as in chromaffin cells[24] but not in other cells[6,31,33], an essential action of NO in pancreatic acinar cells would be to stimulate IP_3 production.

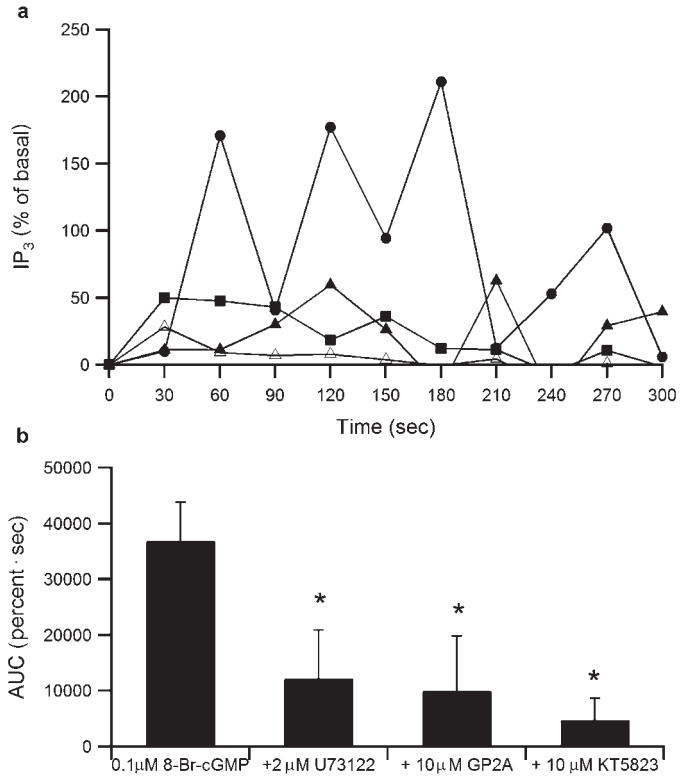

Fig. 4. Effects of U73122, GP2A and KT5823 on 8-Br-cGMP-induced IP_3 production. (a) Time courses of IP_3 production induced by 0.1 μM 8-Br-cGMP in the absence (●) and presence of 2 μM U73122 (■), 10 μM GP2A (△) and 10 μM KT5823 (▲). (b) AUC of 0.1 μM 8-Br-cGMP-induced IP_3 production in the absence and presence of 2 μM U73122, 10 μM GP2A and 10 μM KT5823. Data are means ± SE (n = 3–4). *P < 0.05.

sGC and cGMP in NO-induced IP_3 production

The next question would be whether the cGMP pathway intermediates NO-induced IP_3 production. In many biological systems, it is known that the effects of NO are mediated by cGMP. In the present study, the possible relevance of this cascade was assessed in two ways; first, acini were pretreated with ODQ, a highly selective and irreversible inhibitor of guanylyl cyclase, and we found that SNP-induced IP_3 production was significantly attenuated, indicating that activation of guanylyl cyclase, most likely sGC, by NO is an essential step of the pathway of IP_3 production. Second, instead of SNP, we utilized 8-Br-cGMP to examine if IP_3 production was stimulated by the sGC product, cGMP, and found that this analogue also increased $[IP_3]_i$. This evidence indicated that, in addition to sGC, cGMP production was a subsequent step following sGC activation in NO-induced IP_3 production and the resultant Ca^{2+} response in pancreatic acinar cells. A similar idea was also proposed for parotid acinar cells and chromaffin cells[32,34].

PLC mediates NO-induced IP_3 production

The finding of involvement of cGMP in NO-induced IP_3 production then led us to further investigate how cGMP elicits IP_3 production. It has been previously reported that NO might modulate PLC activity through cGMP[4,37]. We tested this possibility by using U73122 and found that the 8-Br-cGMP-induced increase in $[IP_3]_i$ was significantly inhibited. To our knowledge this is the first evidence to indicate the activation of PLC by cGMP leading to production of IP_3 in pancreatic acinar cells. A similar pathway was also proposed in parotid acinar cells, in which NO could activate PLC, IP_3 synthesis and Ca^{2+} release through cGMP[32].

G-protein is involved in NO-induced IP_3 production

Our findings indicated that NO triggered a rise in cGMP followed by activation of PLC and subsequent IP_3 production and Ca^{2+} release from IP_3-sensitive stores. It is widely recognized that PLC activation is commenced by binding of the α-subunit of the trimeric G-protein molecule. If this step also occurs in the process discussed above, 8-Br-cGMP-induced IP_3 production should be inhibited by G-protein inhibitors. In the current study, we utilized the G-protein antagonist peptide, GP2A. Interestingly, as 8-Br-cGMP-induced IP_3 production was totally abolished in the presence of GP2A, we speculated that NO activated G-protein which is downstream of cGMP formation. The G-protein α-subunit could then bind with PLC, which accelerated IP_3 production and Ca^{2+} release.

PKG participates in NO-induced IP_3 production

The effects of cGMP are known to be mediated by multiple factors; one of which is PKG[29]. To investigate the possible involvement of this kinase, we applied a PKG inhibitor, KT5823, to examine its effect on 8-Br-cGMP-induced IP_3 production. The result showed a marked decrease in cGMP-induced IP_3 production in the presence of KT5823, suggesting that a PKG-dependent pathway was involved in cGMP-stimulated IP_3 production.

Considering these results together, we presume that NO activates sGC and elevates the intracellular cGMP level. This cyclic nucleotide then binds with PKG and affects its catalytic potential, which then could phosphorylate PLC as demonstrated by Huang *et al.*[12] and Xia *et al.*[37], though the end effect reported by them was not potentiation but inhibition of $[Ca^{2+}]_i$. Processing of this signaling finally induces IP_3 production and the resultant $[Ca^{2+}]_i$ increase. Unlike other cells[3,30,36], ADP-ribosylation and/or nitrosylation may be a minor step even if it occurs in pancreatic acinar cells.

In conclusion, the present study is the first to show that an NO-PKG signaling pathway may be coupled to IP_3 production in pancreatic acinar cells and that this cascade is mediated by cGMP/G-protein/PLC activation, which, we think, is a novel signaling pathway functioning between NO and Ca^{2+} mobilization in these cells. This may provide a better understanding of the mechanism of the action of NO in the exocrine pancreas.

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