Full Paper

Two Distinct Effects of cGMP on Cytosolic Ca^{2+} Concentration of Rat Pancreatic β -Cells

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Abstract. The present study investigated the effects of cGMP on cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$) of isolated rat pancreatic β -cells. In the presence of 7.0 mM glucose, NOC 7, a nitric oxide (NO) donor, caused an increase in $[\text{Ca}^{2+}]_c$ of the β -cells, which was abolished by the soluble guanylate cyclase inhibitor ODQ. Similar $[\text{Ca}^{2+}]_c$ elevation was evoked by 8-bromo-cGMP. The $[\text{Ca}^{2+}]_c$ elevating responses to NOC 7 and 8-bromo-cGMP were abolished by nifedipine or in a Ca^{2+} -free medium, but were not affected by thapsigargin, suggesting that they are produced by the Ca^{2+} influx through L-type voltage-operated Ca^{2+} channels. In contrast, NOC 7 and 8-bromo-cGMP decreased the $[\text{Ca}^{2+}]_c$ when it was raised in advance by the elevation of external K^+ concentration to 30 mM or by 4-aminopyridine. The pretreatment with thapsigargin almost abolished the $[\text{Ca}^{2+}]_c$ reduction induced by the agents, suggesting that the action is likely to be primarily attributable to an acceleration of the Ca^{2+} sequestration into the endoplasmic reticulum. These results suggest that cGMP has two distinct effects on the $[\text{Ca}^{2+}]_c$ of rat pancreatic β -cells: a facilitation of the Ca^{2+} influx through L-type voltage-operated Ca^{2+} channels and an acceleration of the Ca^{2+} sequestration in the endoplasmic reticulum.

Keywords: islets of Langerhans, calcium, cGMP, NOC 7, glucose

Introduction

It is well documented that cGMP is implicated as an intracellular signaling molecule that regulates various cellular functions through the activation of a specific cGMP-dependent protein kinase. Nitric oxide (NO) is now assuming physiological importance as an endogenous activator of soluble guanylate cyclase (sGC). Several immunostaining studies have indicated the presence of constitutive NO synthase (NOS) in the pancreatic islet tissues of rats and mice as well as the clonal pancreatic β -cell line HIT-T15 (1–3). Furthermore, NO has been shown to be generated in pancreatic islets in response to glucose (1). These findings suggest a physiological involvement of NO-cGMP system in the regulation of islet function, i.e., hormone secretion.

However, the effect of NO on insulin secretion from β -cells is now a matter of controversy. Several research groups including ours have suggested that NO serves

to increase insulin secretion (1, 4–7), whereas other researchers have proposed that the main effect of NO is to suppress insulin secretion (8–11). Furthermore, the contribution of cGMP to the effects of NO on insulin secretion has not been fully elucidated.

Since it is well established that an increase in cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$) of β -cells triggers insulin secretion (12), the present study investigated effects of NO-cGMP system on the $[\text{Ca}^{2+}]_c$ of isolated rat β -cells. This made it possible to estimate the insulin secretion from the β -cells by monitoring the real-time changes in the $[\text{Ca}^{2+}]_c$ of the cells. The results in the present study show a dual effect of NO-cGMP system on $[\text{Ca}^{2+}]_c$ in rat pancreatic β -cells; i.e., in the presence of 7.0 mM glucose, the NO-cGMP system increases the $[\text{Ca}^{2+}]_c$, whereas it oppositely decreased the $[\text{Ca}^{2+}]_c$ when an elevation of $[\text{Ca}^{2+}]_c$ was produced in advance by 30 mM K^+ or by 4-aminopyridine, a non-selective K^+ channel blocker.

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Materials and Methods

Drugs

The following drugs were used: 4-aminopyridine, 8-bromo-cGMP (8-Br-cGMP), ODQ (1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one), tolbutamide, and thapsigargin (Sigma, St. Louis, MO, USA); collagenase (Yakult, Tokyo); streptomycin and penicillin (Meiji Seika, Tokyo); fetal bovine serum and Bay K 8644 (Wako, Osaka); and fura 2-acetoxymethyl ester (fura 2-AM) and NOC 7 (1-hydroxy-2-oxo-3-[*N*-methyl-3-aminopropyl]-3-methyl-1-triazene), (Dojindo Laboratories, Kumamoto).

ODQ was first dissolved in dimethylsulfoxide. Tolbutamide and NOC 7 were first dissolved in 1 M NaOH. Other drugs were dissolved in distilled water. Each stock solution was further diluted in HEPES-buffered Krebs (HK) solution (129 mM NaCl, 5.0 mM NaHCO₃, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.0 mM CaCl₂, 1.2 mM MgSO₄, 2.8 mM glucose and 10 mM HEPES, adjusted to pH 7.4 with NaOH) just before administration.

Measurement of $[Ca^{2+}]_c$

Pancreatic β -cells were isolated from male Wistar rats (9–12-week-old, 200–300 g; SLC, Hamamatsu). The rats were treated as approved by the Institutional Animal Care and Use Committee and the Guideline of Animal Experiments established by The Japanese Pharmacological Society. The rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p.), and the abdomen was then opened. Collagenase (1.0 mg/ml) dissolved in the HK solution supplemented with 0.1% bovine serum albumin (BSA) was injected into the common bile duct at the distal end after ligation of the duct proximal to the liver. The pancreas was dissected out and incubated at 37°C for 6–14 min. Islets were collected with a micropipette under a stereoscopic microscope and dispersed into single cells in Ca²⁺-free HK solution. The dispersed cells were plated on coverslips and cultured for 1 day in RPMI-1640 medium (Sigma) supplemented with 10% fetal bovine serum, 100 μ g/ml streptomycin, and 100 U/ml penicillin.

$[Ca^{2+}]_c$ was measured in fura 2-loaded cells by dual-wavelength fluorometry. The cells were loaded with 2 μ M fura 2-AM for 30 min at 37°C. They were then mounted in a chamber on the stage of an inverted microscope (Diaphot TMD 300; Nikon, Tokyo) and were continuously superfused with the HK solution maintained at 37°C by use of a peristaltic pump at a flow rate of 1 ml/min. $[Ca^{2+}]_c$ was measured by an Argus-50/CA system (Hamamatsu Photonics, Hamamatsu), with alternating excitation of cells at 340 ± 10 and $380 \pm$

10 nm and monitoring the resultant emission at 510 ± 20 nm. Pairs of 340 and 380 nm fluorescence images were captured every 10 s and were converted to the 340 nm/380 nm ratio images. The 340 nm/380 nm ratio was used to indicate the relative $[Ca^{2+}]_c$ because of the many uncertainties involved in fura 2 calibration (13). Drugs were applied in the superfusing HK solution. β -Cells were selected according to the procedure described previously; i.e., single cells that responded to 7.0 mM glucose and 0.3 mM tolbutamide with increases in $[Ca^{2+}]_c$ were considered to be β -cells (14). We also confirmed by the immunostaining with an anti-insulin antibody (NeoMarkers, Fremont, CA, USA) that more than 90% of the cells isolated from rat islets by the present dispersion technique were β -cells (data not shown).

Results

At 2.8 mM glucose, NOC 7 (1 μ M), an NO donor, caused no changes in $[Ca^{2+}]_c$ of rat pancreatic β -cells (Fig. 1A). Elevating the glucose concentration to 7.0 mM caused an initial decrease and subsequent transient increase in $[Ca^{2+}]_c$, followed by a sustained, moderate elevation of $[Ca^{2+}]_c$ (Fig. 1A). On the sustained elevation of $[Ca^{2+}]_c$ induced by 7.0 mM glucose, NOC 7 (1 μ M) caused an increase in $[Ca^{2+}]_c$ in about 70% of the β -cells tested (Fig. 1A). The NOC 7-induced $[Ca^{2+}]_c$ elevation was abolished by ODQ (10 μ M), a sGC inhibitor (Fig. 1B). A similar $[Ca^{2+}]_c$ elevation was evoked by 8-Br-cGMP (1 mM) at 7.0 mM glucose (Fig. 1C).

The source of Ca²⁺ involved in the $[Ca^{2+}]_c$ -elevating response to NOC 7 at 7.0 mM glucose was next determined. When the cells were exposed to the Ca²⁺-free HK solution for 10 min, NOC 7 (1 μ M) did not cause any increase in the $[Ca^{2+}]_c$ in any of the β -cells tested (Fig. 2A). The $[Ca^{2+}]_c$ -elevating effect of NOC 7 (1 μ M) at 7.0 mM glucose was also abolished by the treatment with nifedipine (1 μ M), a blocker of L-type voltage-operated Ca²⁺ channels, for 10 min (Fig. 2B). In contrast, the pretreatment with thapsigargin (0.3 μ M), an inhibitor of Ca²⁺-ATPase in the endoplasmic reticulum (ER) membrane, for 15 min in the Ca²⁺-free HK solution did not suppress the $[Ca^{2+}]_c$ elevation induced by NOC 7 (1 μ M) at 7.0 mM glucose (Fig. 2: C and D). We confirmed that the pretreatment with thapsigargin (0.3 μ M) abolished the $[Ca^{2+}]_c$ -elevating response to acetylcholine (1 μ M, data not shown).

Elevation of external K⁺ concentration to 30 mM produced a sustained increase in the $[Ca^{2+}]_c$ at 2.8 mM glucose. On this condition, NOC 7 (1 μ M) partially inhibited the $[Ca^{2+}]_c$ elevated by 30 mM external K⁺ (Fig. 3A). Similar $[Ca^{2+}]_c$ reduction was also induced by

Fig. 1. Stimulatory effects of NOC 7 and 8-bromo-cGMP (8-Br-cGMP) on cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) in isolated rat pancreatic β -cells. NOC 7 (1 μ M, A) and 8-Br-cGMP (1 mM, C) were applied in the superfusing solution containing 2.8 (G 2.8) or 7.0 mM (G 7.0) glucose. ODQ (10 μ M), an inhibitor of soluble guanylate cyclase, was present as indicated (B). Horizontal bars indicate the periods when the cells were exposed to the agents specified. The tracings in panels A, B, and C are representative of 33, 10, and 11 cells, respectively, from 2 or 3 rats.

Fig. 2. Source of Ca^{2+} involved in the $[Ca^{2+}]_c$ -elevating response to NOC 7 in isolated rat pancreatic β -cells. NOC 7 (1 μ M) was applied in the Ca^{2+} -free superfusing solution (A) and in the superfusing solution containing nicardipine (NIC, 1 μ M; B) or thapsigargin (TG, 0.3 μ M; C). The superfusing solutions contained 7.0 mM glucose (G 7.0). Intracellular Ca^{2+} stores were depleted by the treatment with TG for 15 min in the Ca^{2+} -free solution and then for an additional 10 min in the normal solution (C). Horizontal bars indicate the periods when the cells were exposed to the agents specified. The tracings in panels A, B, and C are representative of 18, 21, and 11 cells, respectively, from 2 rats. D: Summarized data showing effects of NOC 7 on $[Ca^{2+}]_c$ before (open bar) and after the treatment with TG (0.3 μ M, closed bar) depicted in the panel C. The changes in $[Ca^{2+}]_c$ are expressed as a percentage of that induced by 7.0 mM glucose. Values are means $\pm$ S.E.M. for the data of 11 cells.

Fig. 3. Inhibitory effects of NOC 7 and 8-bromo-cGMP (8-Br-cGMP) on $[Ca^{2+}]_c$ of rat pancreatic β -cells. NOC 7 (1 μ M, A and C) and 8-Br-cGMP (1 mM, B) were applied in the superfusing solution containing 30 mM external K^+ (30K), 30 K plus thapsigargin (TG, 0.3 μ M), or 4-aminopyridine (4-AP, 10 mM) at 2.8 mM glucose (G 2.8). It was confirmed in the same preparation that NOC 7 (1 μ M) elevated $[Ca^{2+}]_c$ at 7.0 mM glucose. Horizontal bars indicate the periods when the cells were exposed to the agents specified. The tracings in panels A, B, and C are representative of 14, 5, and 17 cells, respectively, from 2 or 3 rats. D: Summarized data showing effects of NOC 7 (1 μ M) and 8-Br-cGMP (1 mM) on the $[Ca^{2+}]_c$ elevated by 30 K in the absence (open bar) and the presence of TG (0.3 μ M, closed bar) depicted in panels A and B. The changes in $[Ca^{2+}]_c$ are expressed as a percentage of the amplitude of the increase in $[Ca^{2+}]_c$ induced by 30 K. Values are means $\pm$ S.E.M. for the data of 14 (NOC 7) and 5 cells (8-Br-cGMP). ** $P < 0.01$, * $P < 0.05$.

8-Br-cGMP (1 mM) in the presence of 30 mM external K^+ (Fig. 3B). The pretreatment with 0.3 μ M thapsigargin almost abolished the $[Ca^{2+}]_c$ reduction induced by NOC 7 (1 μ M, Fig. 3: A and D) and 8-Br-cGMP (1 mM, Fig. 3: B and D) in the presence of 30 mM external K^+ . Similarly, NOC 7 (1 μ M) reduced the $[Ca^{2+}]_c$, when $[Ca^{2+}]_c$ was elevated in advance by 4-aminopyridine (10 mM), a non-selective K^+ channel blocker (Fig. 3C).

Discussion

NO serves numerous biological functions, some of which are mediated by the ability of NO to activate sGC,

resulting in cGMP formation (15, 16). The present study demonstrated that the NO donor NOC 7 (1 μ M) caused an increase in $[Ca^{2+}]_c$ of rat pancreatic β -cells at 7.0 mM glucose, which was produced by the Ca^{2+} influx through L-type voltage-operated Ca^{2+} channels. The stimulatory effect of NOC 7 was likely to be mediated by cGMP, because it was abolished by the sGC inhibitor ODQ and similar $[Ca^{2+}]_c$ responses were produced by 8-Br-cGMP. These results suggest that cGMP is a second messenger in NO signal transduction, which stimulates the Ca^{2+} influx into the β -cells. Although insulin secretion is not always proportional to $[Ca^{2+}]_c$ (17), it is well established that the Ca^{2+} influx into β -cells triggers

insulin secretion (12). Therefore, the cGMP-induced increase in $[Ca^{2+}]_c$ might be expected to potentiate the glucose-induced insulin secretion. This is in good accordance with previous studies showing that the glucose-induced insulin secretion was facilitated by exogenous cGMP analogs (18, 19) and by aqueous NO solution or NO donors (1, 4–7). In contrast, several studies revealed that NO donors inhibited the glucose-induced insulin secretion (8–11). However, some of these studies clearly showed that in contrast to the NO donors, membrane-permeable cGMP analogs facilitated the glucose-induced insulin secretion (9, 10). Therefore, the inhibitory effect of NO on the hormone secretion does not appear to be mediated by cGMP.

The increase in $[Ca^{2+}]_c$ induced by NOC 7 and 8-Br-cGMP at 7.0 mM glucose was abolished in a Ca^{2+} -free medium and by nifedipine, a blocker of L-type voltage-operated Ca^{2+} channels, suggesting that the response was mediated by a Ca^{2+} influx through L-type voltage-operated Ca^{2+} channels. It is unlikely that the Ca^{2+} release from the ER was involved in the $[Ca^{2+}]_c$ elevating response to NOC 7 and 8-Br-cGMP, because the response was not affected by the pretreatment with thapsigargin. These results are apparently not consistent with those in the previous study showing that the NO-induced increase in $[Ca^{2+}]_c$ of isolated rat β -cells was completely abolished when cells were pretreated with 10 μ M thapsigargin (6). However, the concentration of thapsigargin (10 μ M) used in the previous study was much higher than that used in the present study (0.3 μ M). We believe that 0.3 μ M thapsigargin was fully effective to inhibit the ER Ca^{2+} -ATPase, since the same concentration of thapsigargin abolished the acetylcholine-induced $[Ca^{2+}]_c$ elevation. Similar concentrations of thapsigargin, i.e., 0.2 to 0.5 μ M, have also been shown to inhibit the acetylcholine-evoked $[Ca^{2+}]_c$ response in rat β -cells (20). It might be possible that high concentration of thapsigargin inhibited the NO-induced $[Ca^{2+}]_c$ elevation through a mechanism other than the ER Ca^{2+} -ATPase inhibition, e.g., inhibition of capacitative Ca^{2+} entry and L- and T-type voltage-operated Ca^{2+} channels (21), although the precise reason for the discrepancy is presently not clear.

When the $[Ca^{2+}]_c$ of the β -cells was in advance elevated by 30 mM K^+ or by 4-aminopyridine, NOC 7 and 8-Br-cGMP reduced the $[Ca^{2+}]_c$. The cGMP-induced $[Ca^{2+}]_c$ reduction was almost abolished by the pretreatment with thapsigargin, which irreversibly inhibits Ca^{2+} -ATPase in the ER membrane without affecting the plasma membrane Ca^{2+} -ATPase (22). Therefore, the $[Ca^{2+}]_c$ reducing action of cGMP is likely to be due primarily to the acceleration of the Ca^{2+} sequestration in the ER. The activation by cGMP of Ca^{2+} -ATPase

on the ER is also observed in other types of cells such as smooth muscle cells (13). Although the physiological significance of the $[Ca^{2+}]_c$ reducing action of cGMP is not clear, the nucleotide may play a role in protecting the cells from an excessive rise in $[Ca^{2+}]_c$. Sustained $[Ca^{2+}]_c$ elevation has been shown to promote apoptotic cell death in pancreatic β -cells (23). It may also be possible that the cGMP-stimulated Ca^{2+} sequestration augments the action of insulinotropic substances that cause the release of Ca^{2+} from the ER, e.g., acetylcholine (20).

In the presence of 7.0 mM glucose, cGMP increased the $[Ca^{2+}]_c$ of the β -cells by facilitating the Ca^{2+} influx through L-type voltage-operated Ca^{2+} channels. Since glucose depolarizes the membrane of β -cells by inhibiting K_{ATP} channels (24), it is possible that cGMP augments the membrane depolarization induced by glucose. When β -cells were depolarized by 30 mM K^+ or by 4-aminopyridine, the activity of cGMP to increase the $[Ca^{2+}]_c$ was impaired. Increasing the external K^+ concentration to 30 mM is expected to reduce the K^+ efflux through K^+ channels by reducing the K^+ gradient across the cell membrane, and 4-aminopyridine is a non-selective K^+ channel blocker. This implies the contribution of K^+ channels to the cGMP-induced increase in $[Ca^{2+}]_c$. Taken together, it is suggested that cGMP inhibits K^+ channels sensitive to 4-aminopyridine, thereby depolarizing the membrane of the β -cells. Further investigation using the patch clamp technique would be required to characterize the K^+ channels contributing to the effect of cGMP in the β -cells.

In conclusion, cGMP has two distinct effects on $[Ca^{2+}]_c$ of rat pancreatic β -cells, i.e., a facilitation of the Ca^{2+} influx through L-type voltage-operated Ca^{2+} channels and an acceleration of the Ca^{2+} sequestration in the endoplasmic reticulum. The former seems to be caused by the inhibition of K^+ channels. It could be proposed that the NO-cGMP system functions as a physiological regulator of the insulin secretion from the pancreatic β -cells.

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