Role of calcium mobilization in sodium nitroprusside-induced increase of calcium-activated potassium currents in gastric antral circular myocytes of guinea pig[1]

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ABSTRACT

AIM: To investigate the role of calcium mobilization in the calcium-activated potassium currents [$I_{K(Ca)}$] increased by sodium nitroprusside (SNP), a nitric oxide (NO) donor, in gastric antral circular myocytes of the guinea pig. **METHODS:** A perforated patch-clamp technique was used, and the myocytes were isolated by collagenase. **RESULTS:** SNP 100 μmol/L significantly increased $I_{K(Ca)}$, and enhanced the spontaneous transient outward currents (STOC). SNP-induced increase of $I_{K(Ca)}$ was not blocked by extracellular calcium-free solution (containing egtazic acid 10 μmol/L and nicardipine 5 μmol/L, an L-type calcium channel blocker. And SNP 100 μmol/L suppressed the L-type calcium currents (I_{Ca}). SNP-induced increase of STOC was inhibited by heparin 3 g/L, a potent inhibitor of inositol triphosphate receptor ($InsP_3R$). However, ryanodine 10 μmol/L, an inhibitor of calcium-induced calcium release (CICR), did not inhibit the effect of SNP-induced increase of STOC. Methylene blue (1 μmol/L), an inhibitor of soluble guanylate cyclase, also inhibited such an effect. **CONCLUSION:** The increase of $I_{K(Ca)}$ caused by SNP may be mediated by cGMP via IP_3-sensitive calcium pools, however, extracellular Ca^{2+} may not be involved in the process.

INTRODUCTION

The mechanism by which nitric oxide (NO) relaxes smooth muscle cells has long been a controversial topic and is not yet fully understood[1]. Previous studies showed that as one of the mediators of non-adrenergic, non-cholinergic (NANC) nerves in gastrointestinal (GI) smooth muscle, NO mediated the inhibitory junction potentials (IJP) and induced hyperpolarization of the cell membrane[2]. NO and NO donors, such as sodium nitroprusside (SNP) and 3-morpholino-sydnonimine-hydrochloride (SIN-1), are generally thought to cause smooth muscle relaxation by activating guanosine 3',5'-cyclic monophosphate (cGMP)[3]. Presently several possibilities about the mechaninsm of relaxation of smooth muscle by NO and cGMP are a matter of debate: 1) hyperpolarization of cell membrane, 2) sequestration of Ca^{2+} with lowered cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), 3) reduced sensitivity of the contractile apparatus, *ie*, of myosin light-chain kinase, and 4) reduced activation of second messengers involved in triggering smooth muscle contraction.

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In our previous study, we have reported that NO increased calcium-activated potassium currents in gastric antral circular myocytes of guinea pig, and the effect of NO on $I_{K(Ca)}$ may be mediated by cGMP[4]. Recent patch-clamp studies also demonstrated that NO-donors enhanced $I_{K(Ca)}$ in several GI smooth muscle cells[5-7]. However, the mechanism of how NO and NO-donors increased $I_{K(Ca)}$ is not yet fully understood. $I_{K(Ca)}$ could be triggered by a Ca^{2+} -induced Ca^{2+} release (CICR) mechanism. Hyperpolarization of the cell membrane and receptor-bound mechanisms are able to regulate $[Ca^{2+}]_i$, which is an important regulator of contraction in smooth muscle. For example, voltage-operated L-type calcium channels increased $[Ca^{2+}]_i$ with depolarization and decreased $[Ca^{2+}]_i$ with hyperpolarization[9]. NO modulated the intracellular $[Ca^{2+}]_i$ through the IP_3-induced calcium release (IICR)[10]. The studies by Petkov *et al*[11] also showed that NO-induced relaxation was due to an increase of Ca^{2+} accumulation in the sarcoplasmic reticulum, which in turn led to activation of the vectorial Ca^{2+} release from the sarcoplasmic reticulum toward the plasmalemma in guinea pig gastric fundus smooth muscle. Therefore it is not fully clear about the mobilization of calcium in the SNP-induced increase of $I_{K(Ca)}$ in gastrointestinal smooth muscle cell.

The aim of the present study is to investigate the role of intracellular and extracellular calcium in SNP-induced increase of $I_{K(Ca)}$ in gastric antral circular myocytes of guinea pigs.

MATERIALS AND METHODS

Preparation of cells Guinea pigs of either sex, weighing 250-350 g, were euthanized by lethal intravenous injection of sodium pentobarbital (50 mg/kg). The antral part of the stomach was rapidly cut, then the muscosal layer was separated from the muscle layers. The longitudinal layer of muscle was then dissected from the other muscle layers using fine scissors, and then cut into small segments (1 mm×4 mm). These segments were kept in the modified Kraft-Bruhe (K-B) medium at 4 °C for 15 min. Then they were incubated at 36 °C in 4 mL of digestion medium [Ca-free Physiologic Salt Solution (Ca-free PSS)] containing 0.1 % collagenase II, 0.1 % dithioerythreitol, 0.15 % trypsin inhibitor, and 0.2 % bovine serum albumin for 25-35 min. The softened muscle segments were transferred into the modified K-B medium, and the single cells were dispersed by gentle trituration with a wide-bore fire-polished glass pipette. The isolated gastric myocytes were kept in the modified K-B medium at 4 °C for use.

Electrophysiologic recording Isolated cells were transferred to a 0.1 mL chamber on the stage of an inverted microscope (IX-70 Olympus, Japan) and allowed to settle for 10-15 min. Then the cells were continuously perfused with isosmotic physiologic salt solution at a rate of 0.9-1.0 mL/min. An 8-channel perfusion system (L/M-sps-8, List Electronics, Germany) was used to exchange the solution. The $I_{K(Ca)}$ was recorded using the perforated patch-clamp mode of the patch-clamp technique[12]. Patch-clamp pipettes were manufactured from borosilicate glass capillaries (GC 150T-7.5, Clark Electromedical Instruments, UK) using a two-stage puller (PP-83, Narishige, Japan). The resistance of patch pipette filled with pipette solution was 3-5 MΩ. Liquid junction potentials were canceled prior to seal formation. Whole-cell currents were recorded with an Axopatch 1-D patch-clamp amplifier (Axon Instrument, USA) and data were filtered at 1 kHz. Command pulses, data acquisition and storge were applied using the IBM-compatible 486-grade computer and pCLAMP 6.02 software. Spontaneous transient outward currents (STOC) were recorded simultaneously by polygraph (RM6200, Nihon kohden, Tokyo, Japan). All experiments were performed at room temperature (20-25 °C). After the $I_{K(Ca)}$ reached a steady state the cells were exposed to SNP in perforated whole-cell patch-clamp mode. $I_{K(Ca)}$ started to increase at (4.2±2.9) s, it reached pick values at (11±4) s and completely recovered at (297±87) s after washing out.

Drugs and solutions Tyrode's solution contained (mmol/L) NaCl 147, KCl 4, $MgCl_2 \cdot 6H_2O$ 1.05, $CaCl_2 \cdot 2H_2O$ 0.42, $Na_2PO_4 \cdot 2H_2O$ 1.81, and glucose 5.5; pH was adjusted to 7.35 with NaOH. Physiological salt solution contained (PSS, mmol/L) NaCl 134.8, KCl 4.5, $MgCl_2 \cdot 6H_2O$ 1.0, $CaCl_2 \cdot 2H_2O$ 2.0, glucose 5.0, and HEPES 10.0, pH was adjusted to 7.4 with Tris [hydrocymethyl] aminomethane (TRIZMA). In Ca^{2+}-free PSS, $CaCl_2 \cdot 2H_2O$ 2.0 mmol/L was omitted from PSS. Modified K-B solution contained (mmol/L) *L*-glutamate 50, KCl 50, taurine 20, KH_2PO_4 20, $MgCl_2 \cdot 6H_2O$ 3.0, glucose 10.0, HEPES 10.0, and egtazic acid 0.5; pH was adjusted to 7.4 with KOH. And in order to abolish delayed rectifier potassium currents [$I_{K(V)}$], external solution contained 4-aminopyridine 10 mmol·L^{-1} (4-AP), a kind of selective inhibiter of $I_{K(V)}$. The pipette solution recording $I_{K(Ca)}$ contained (mmol/L) potassium-aspartic acid 110, Mg-ATP 5, HEPES 5, $MgCl_2 \cdot 6H_2O$ 1.0, KCl 20, egtazic acid 0.1, di-*tris*-

creatine phoshate 2.5, and disodium-creatine phosphate 2.5; and its pH was adjusted to 7.3 with KOH. The pipette solution recording I_{Ca} contained CsCl 135, $MgCl_2 \cdot 6H_2O$ 4.0, HEPES 10, Na_2ATP 2, egtazic acid 10, and tetraethylammonium (TEA) 20; pH was adjusted to 7.35 with Tris. Nystatin was dissolved in pure dimethyl sul-phoxide (Me_2SO) at 100 g/L and kept at 0 °C. In experiment, nystatin 200 mg/L was added to the pipette solution.

SNP was obtained from Nakarae Chemicals Ltd (Japan). Ryanodine was from Vago Chemicals Ltd (Japan). Other reagents were purchased from Sigma (USA). All stock solutions were kept at 4 °C and diluted in PSS before experiment.

Data analysis Data were expressed as mean ±SEM. Statistics were made by Student's *t*-test when appropriate.

RESULTS

Effect of SNP on $I_{K(Ca)}$ in guinea pig gastric myocytes Under the perforated patch-clamp mode, the membrane potential was clamped at –60 mV, and $I_{K(Ca)}$ was elicited by step voltage command pulse from –40 mV to 100 mV for 400 ms with step of 20 mV at 10-s intervals. The amplitude of $I_{K(Ca)}$ was (1.5±0.9) nA at +60 mV (n=50). In statistics, peak values of $I_{K(Ca)}$ at every level of membrane potential were normalized to the values at +80 mV or +100 mV in control condition. SNP 100 μmol/L caused an efficient increase in amplitude of $I_{K(Ca)}$ by 21 %±5 % at +60 mV (n=13, Fig 1). In perforated patch-clamp mode and same pipette solution, the holding potential was clamped at -20 mV, the spontaneous transient outward currents (STOC) were then recorded. In order to determine the STOC , the component of $I_{K(Ca)}$, the effects of TEA, a nonselective potassium channel blocker, and charybdotoxin (ChTX), a selective calcium-activated potassium channel blocker, on STOC were observed. In the present study, STOC were markedly inhibited by TEA 4 mmol/L and ChTX 200 nmol/L (Fig 2A, 2B, respectively, n= 11). SNP 100 μmol/L obviously increased the amplitude of STOC (Fig 2).

Effect of extracellular Ca^{2+} on SNP-induced increase of $I_{K(Ca)}$ It is well known that $I_{K(Ca)}$ is activated by intracellular free calcium, and that extracellular calcium is necessary for efficiently controlling intracellular calcium homeostasis. In order to determine the possibility of Ca^{2+} influx involved in SNP-induced increase of $I_{K(Ca)}$, we removed extracellular Ca^{2+} and observed

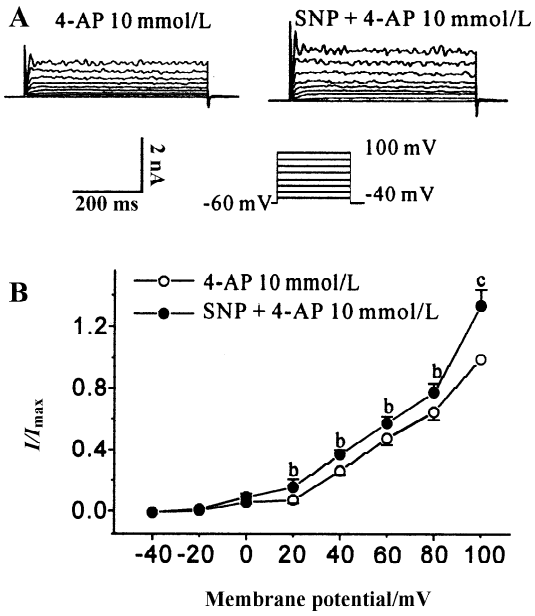

Fig 1. Effect of sodium nitroprusside 100 μmol/L on the $I_{K(Ca)}$ in the gastric antral circular myocytes of guinea-pig. A) The raw traces of $I_{K(Ca)}$ before and after the administration of SNP 100 μmol/L. B) *I/V* relationship of $I_{K(Ca)}$ before and after the administration of SNP 100 μmol/L. Peak values were normalized to the values obtained at +100 mV in control condition. n=13. Mean±SEM. $^bP<0.05$, $^cP<0.01$ *vs* 4-AP 10 mmol/L.

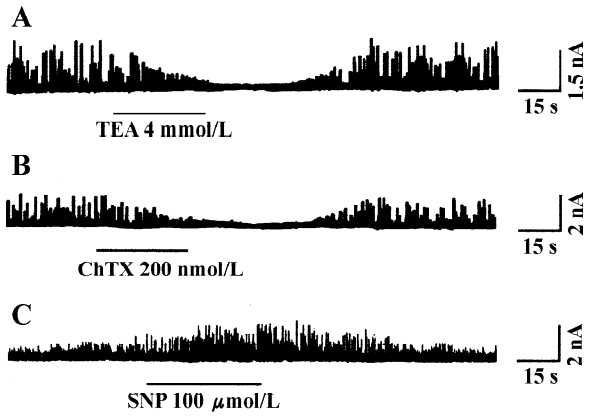

Fig 2. Effects of TEA, ChTX, and SNP on STOC. n=11.

the effect of SNP on $I_{K(Ca)}$. When Ca^{2+}-free solution (containing egtazic acid 10 μmol/L) was replaced with Ca^{2+}-free SNP solution, $I_{K(Ca)}$ was also significantly increased by 22 %±7 % (n=8, Fig 3A, 3B). Similar result was also found in STOC. After perfusion with external Ca^{2+}-free solution for about 6 min, STOC were obviously decreased, but not abolished. However, external Ca^{2+} free solution did not inhibit SNP-induced increase of STOC (Fig 3C). In the present study, $I_{K(Ca)}$ was

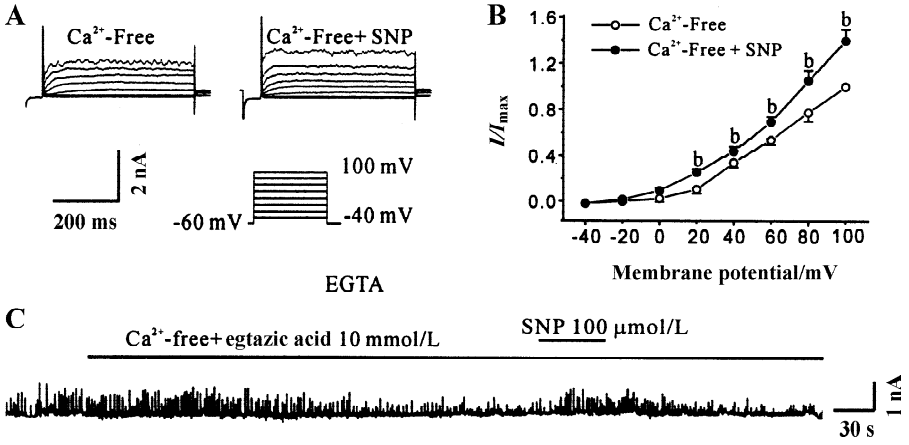

Fig 3. Effect of external Ca^{2+}-free solution on SNP-induced increase of $I_{K(Ca)}$. A) The raw traces before and after SNP 100 μmol/L was added in external Ca^{2+}-free solution. B) *I/V* relationship of $I_{K(Ca)}$ for cells exposed to SNP 100 μmol/L in Ca^{2+}-free solution. Peak values in Ca^{2+}-free solution containing SNP 100 μmol/L were normalized to the values obtained at +100 mV in Ca^{2+}-free solution. *n*=8. Mean±SEM. $^bP<0.05$ *vs* Ca^{2+}-free solution. C) Effect of external Ca^{2+}-free solution on SNP-induced increase of STOC. *n*=8.

markedly inhibited by nicardipine (5 μmol/L), an L-type calcium channel blocker, but nicardipine did not block SNP-induced increase of $I_{K(Ca)}$ (*n*=7, Fig 4). These results suggested that extracelluar Ca^{2+} was not involved in SNP-induced increase of $I_{K(Ca)}$.

Effect of SNP on the L-type calcium currents To determine whether SNP could relax the gastric smooth muscle through inhibiting the Ca^{2+} influx, we evaluated the effect of SNP on I_{Ca}. SNP 100 μmol/L markedly suppressed I_{Ca} by 47.2 %±12.3 % (*n*=9, Fig 5). The result was similar with that in opossum esophageal circular muscle cells[13].

Effect of intracellular Ca^{2+} on SNP-induced increase of $I_{K(Ca)}$ In order to determine the role of intracellular Ca^{2+} release in SNP-induced increase of $I_{K(Ca)}$, we investigated intracellular calcium-releasing pathway, the calcium-induced calcium release (CICR) or the inositol triphosphate-induced calcium release (IICR). $I_{K(Ca)}$ is activated by intracellular Ca^{2+} and can be monitored by STOC. STOC are caused by the cyclical release of calcium from stores close to the membrane[14], so they were observed in order to examine the effect of intracellular Ca^{2+} on SNP-induced increase of $I_{K(Ca)}$. SNP inhibited L-type calcium current (Fig 5). Heparin 3 g/L, an IP_3 receptor blocker in calcium store membrane, markedly decreased STOC, and SNP 100 μmol/L could not increase STOC after pretreatment with heparin 3 g/L (Fig 6A, *n*=7). In the present study, ryanodine 10 μmol/L, a CICR inhibitor, increased STOC at first, and after about 8 min, STOC were markedly

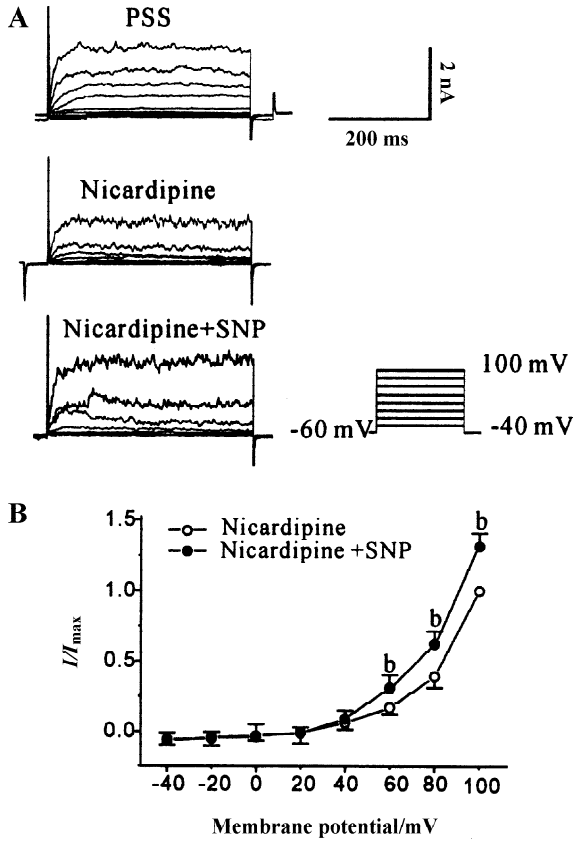

Fig 4. Effect of nicardipine 5 μmol/L on SNP-induced increase of $I_{K(Ca)}$. A) The raw traces obtained in the condition of control (PSS), nicardipine 5 μmol/L, and nicardipine 5 μmol/L+SNP 100 μmol/L. B) *I/V* relationship of $I_{K(Ca)}$ for cells exposed to SNP 100 μmol/L in presence of nicardipine 5 μmol/L. Peak values in nicadipine and SNP group were normalized to the values obtained at +100 mV in nicardipine group. *n*=8. Mean±SEM. $^bP<0.05$ *vs* nicardipine group.

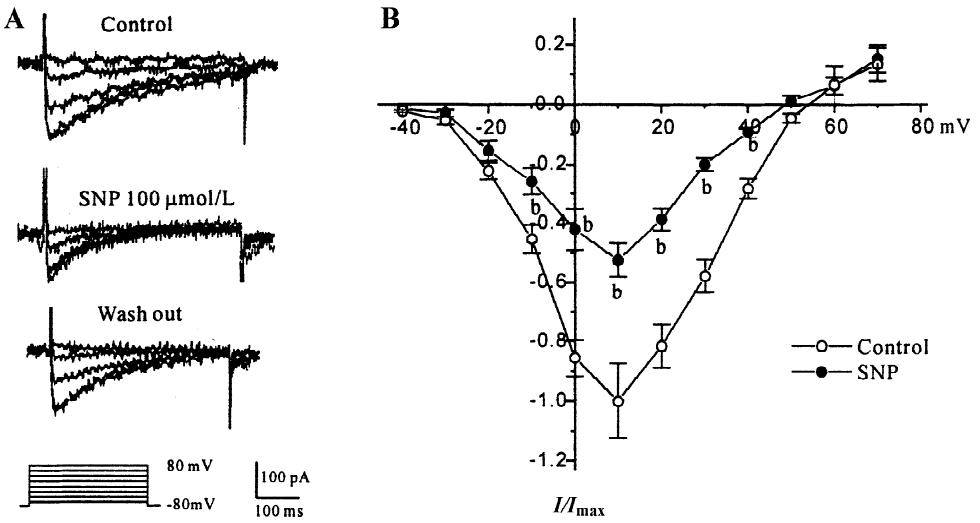

Fig 5. Effect of SNP 100 μmol/L on I_{Ca} in gastric antral circular myocytes of guinea pigs. A) The raw traces obtained in the condition of control (PSS), SNP 100 μmol/L, and washout. B) *I/V* relationship of I_{Ca} for cells exposed to SNP 100 μmol/L. *n*=9. Mean±SEM. $^{b}P<0.05$ *vs* control.

inhibited by ryanodine, and STOC could not be increased by caffeine 1 mmol/L, an agonist for CICR. However, SNP 100 μmol/L markedly increased the STOC after the CICR was sufficiently depleted by ryanodine 10 μmol/L (Fig 6B, *n*=10).

Effect of SNP on STOC in the present of methylene blue In order to determine whether the effect of SNP on STOC is mediated by cGMP, the effect of methylene blue on SNP-induced increase of STOC was observed. SNP 100 μmol/L could not increase STOC after pretreatment with methylene blue (1 μmol/L) for 3 min (Fig 6C, *n*=8).

DISCUSSION

In the present study, we found that SNP-induced increase of $I_{K(Ca)}$ was not inhibited by external Ca^{2+}-free solution and nicardipine. And L-type calcium current was inhibited by SNP, however, STOC was increased by SNP. The effect of SNP on STOC was markedly

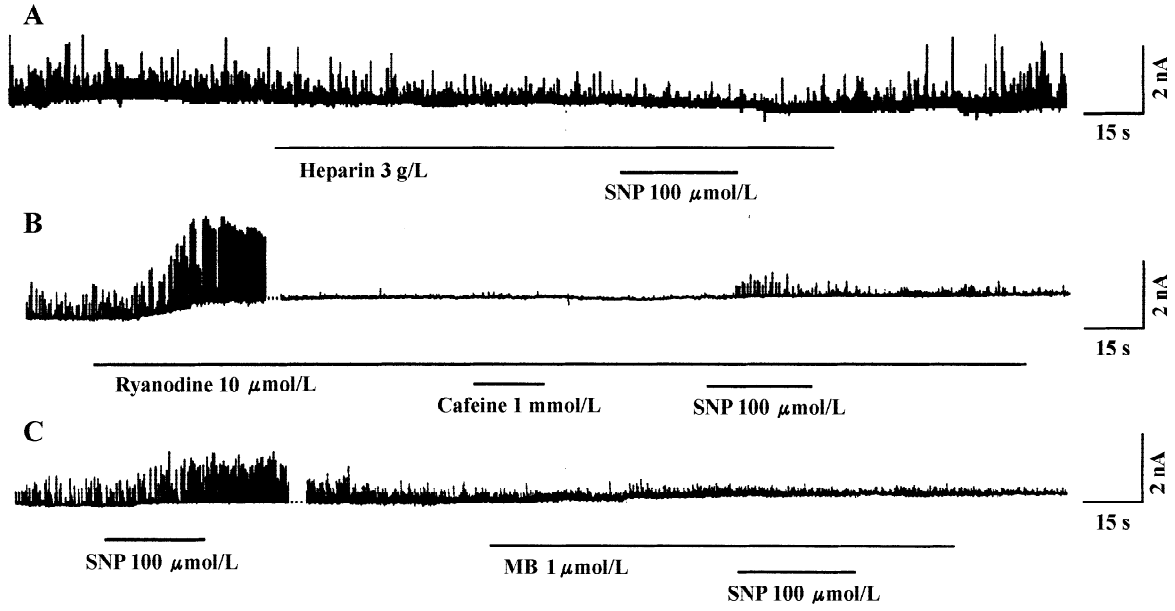

Fig 6. Effect of intracellular Ca^{2+} pool and cGMP on SNP-induced increase of STOC. A) Heparin 3 g/L inhibited SNP-induced increase of STOC. *n*=7. B) Ryanodine 10 μmol/L did not block SNP-induced increase of STOC. *n*=10. C) Methylene blue 1 μmol/L inhibited SNP-induced increase of STOC. *n*=8.

inhibited by heparin, but not inhibited by ryanodine. Methylene blue could inhibit SNP-induced increase of STOC.

It is well known that $I_{K(Ca)}$ plays a very important role in relaxation of gastrointestinal smooth muscle. Many experiments have shown that $I_{K(Ca)}$ was increased by NO and NO donors in a variety of cells, such as in the colonic smooth muscle of rabbits[5], opossum esophageal smooth muscle[15], and single myocytes of the guinea-pig proximal colon[7]. In the present study, we confirmed that $I_{K(Ca)}$ was increased by SNP in gastric antral circular myocytes of the guinea pig. In our previous study, the depolarization pulse activated two types of K^+ currents in gastric antral circular myocytes of guinea pig, calcium-activated potassium currents [$I_{K(Ca)}$] and delayed rectified potassium currents [$I_{K(V)}$][16]. In order to abolish $I_{K(V)}$, we used three ways in this study. (i) The pipette solution contained egtazic acid 0.1 mmol/L; (ii) The perfusing solution recording $I_{K(Ca)}$ contained 4-AP 10 mmol/L, a kind of nonselective inhibitor of $I_{K(V);}$ (iii) STOC were significantly blocked by TEA and ChTX, while they were potentiated by SNP (Fig 2).

The significance of the present study is to determine the relationship between NO-induced increase of $I_{K(Ca)}$ and Ca^{2+} signaling in gastric antrial circular myocytes. It has already been described that Ca^{2+} signaling activated by NO is an important factor for regulating the contraction of smooth muscle. Petkov *et al*[11] found that L-type Ca^{2+} channel was involved in the process which NO donors elicited CICR and hyperpolarization. Watson *et al*[17] described that NO donors-induced hyperpolarizations were dependent on external Ca^{2+}, but not on the influx of Ca^{2+} through either ' L' - or ' N' -type Ca^{2+} channel. In order to determine the pathway of increase in $[Ca^{2+}]_i$ which is involved in triggering $I_{K(Ca)}$ activation, we performed different methods. In our experiment, under the external calcium-free condition, $I_{K(Ca)}$ was increased by SNP (Fig 3 A, B) and STOC were also potentiated by SNP (Fig 3C). Although STOC were obviously inhibited by external Ca^{2+}-free solution, however, SNP still increased STOC. Similar results were obtained from pulmonary vascular smooth muscle cell[18]. It is well known that there are six subtypes of voltage-dependent Ca^{2+} channels: L-, N-, P-, Q-, R-, and T-type. In smooth muscle, only the L-type channel is considered to be a major Ca^{2+} influx pathway[19]. Petkov *et al*[11] found that NO donors elicited membrane hyperpolarization and relaxation via L-type Ca^{2+} current. However, in our present study, nicardipine, one of L-type calcium channel blockers did not block SNP-induced increase of $I_{K(Ca)}$. Similar results were obtained in the experiments of guinea pig aorta[20]. These data suggest that SNP-induced increase of $I_{K(Ca)}$ was not dependent on extracellular Ca^{2+}. In our present study, SNP inhibited L-type calcium current (Fig 5). The result is similar to opossum esophageal circular muscle cells[13]. It may be one of the reasons that NO suppressed the contraction of smooth muscle.

It is suggested that NO and NO donors exert their relaxant effects on smooth muscles by altering the intracellular Ca^{2+} homeostasis[6,21]. Jury *et al* [22] concluded that NO release activated K^+ outward currents, which depended on Ca^{2+} release from the SR stores. However, there were different viewes on the intracellular Ca^{2+} mobilization by NO and NO donors. Some studies showed that NO donor-induced increase of $I_{K(Ca)}$ could be triggered by a Ca^{2+} -induced Ca^{2+} release (CICR) mechanism[11,23]. Other studies showed that NO modulated the intracellular $[Ca^{2+}]_i$ through the IP_3-induced calcium release[24,10]. In our present study, heparin completely blocked SNP-induced increase of $I_{K(Ca)}$, while ryanodine did not inhibit such effect (Fig 6A,B). Our data suggest that SNP-induced increase of $I_{K(Ca)}$, may be caused by IICR in gastric antral circular myocytes of the guinea pig.

It is well known that NO- and NO donor-induced increase of $I_{K(Ca)}$ was mediated directly[23] or indirectly via cGMP and cyclic GMP-dependent kinase[25,26]. In the esophageal body, methylene blue was effective in inhibiting the hyperpolarizing responses to SIN-1 and SNP[27,28]. The inhibitory effect could occur by a mechanism of inhibiting guanylate cyclase[29]. However, inhibition of Ca^{2+} release resulted from the generation of superoxide anions by methylene blue[15], is also a possible mechanism of the inhibitory effect of methylene blue on NO-mediated hyperpolarizations and SNP-induced increase of $I_{K(Ca)}$. In the previous study, we performed further experiments to investigate the effect of cGMP on SNP-induced increase of STOC. As shown in Fig 6C, SNP 100 μmol/L could not increase STOC after pretreatment with methylene blue 1 μmol/L for about 3 min. These data suggest that the intracellular calcium release involved in SNP-induced increase of $I_{K(Ca)}$ may be mediated by cGMP.

In summary, our study showed that SNP increased $I_{K(Ca)}$ in gastic antral circular myocytes of guinea-pig. The increase of $I_{K(Ca)}$ caused by SNP may be triggered by cGMP through IP_3-sensitive calcium pools, however,

extracellular Ca^{2+} was not involved in the process.

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