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# Effects of nitroprusside, 3-morpholino-sydnonimine, and spermine on calcium-sensitive potassium currents in gastric antral circular myocytes of guinea pig[1]

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**KEY WORDS** pyloric antrum; patch-clamp techniques; nitroprusside; sydnones; spermine; tetraethylammonium; methylene blue

## ABSTRACT

**AIM**: To determine the effect of nitric oxide (NO) on calcium-activated potassium currents in antral circular myocytes of the guinea pig stomach. **METHODS**: The whole-cell patch-clamp techniques were used, and the cells were isolated by collagenase. Sodium nitroprusside (SNP), spermine, and 3-morpholino-sydnonimine (SIN-1) were used as nitric oxide donors. **RESULTS**: Outward potassium currents were remarkably inhibited by tetraethylammonium (TEA) 1 mmol·L$^{-1}$. Charybdotoxin 200 nmol·L$^{-1}$, a specific inhibitor of $K_{Ca}$ channel, also greatly inhibited $I_{K(Ca)}$. NO donors remarkably increased $I_{K(Ca)}$ in guinea pig gastric antral circular myocytes with perforated patch-clamp techniques but not conventional whole-cell patch-clamp techniques. Spermine 100 μmol·L$^{-1}$ increased $I_{K(Ca)}$ by 14 % ± 10 % (at 60 mV, $P < 0.01$), SIN-1 200 μmol·L$^{-1}$ increased $I_{K(Ca)}$ by 19 % ± 14 % (at 60 mV, $P < 0.01$), and SNP 100 μmol·L$^{-1}$ increased $I_{K(Ca)}$ by 24 % ± 13 % (at 60 mV, $P < 0.01$) respectively. SIN-1- and SNP-induced increase of $I_{K(Ca)}$ was blocked by methylene blue 1 μmol·L$^{-1}$. **CONCLUSION**: NO increases 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 cyclic GMP.

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## INTRODUCTION

In our previous study, we have reported that exogenous nitric oxide (NO) inhibits gastric antral muscle motility in rat *in vitro*[1]. However, its ionic mechanism is not yet fully understood. A number of reports indicated that calcium-activated potassium current [$I_{K(Ca)}$] participated in NO-induced gastrointestinal inhibition[2-4]. Lu *et al*[2] have observed that NO increases whole cell outward $K^+$ current by activating $K_{Ca}$ channels of rabbit colon smooth muscle cells through a cyclic GMP pathway. Recently, it has been reported that sodium nitroprusside (SNP) directly increases the number of active large conductance of calcium-activated potassium channels (BK channels) in the guinea pig colon smooth muscle cell[3]. On the contrary, Zhang *et al*[4] have suggested that NO-mediated hyperpolarization may be produced by suppression of a $Ca^{2+}$-stimulated $Cl^-$ conductance in opossum esophageal smooth muscle. The effect of NO on $K_{Ca}$ channel activity of gastric antral myocytes, however, is currently unknown. In the present study, to determine the effect of NO on $I_{K(Ca)}$ in gastric antral circular smooth muscle cells (GACSMC) of the guinea pig, the effects of NO donors, SNP, SIN-1, and spermine, were investigated on $I_{K(Ca)}$ using the conventional patch-clamp and perforated patch-clamp techniques.

## MATERIALS AND METHODS

**Preparation of cells** EWG/B guinea pigs, bred in the Experimental Animal Department of Norman Bethune University, Certificate № 10-6004, either sex, weighing 250 - 350 g were used. Single smooth muscle cells were isolated from the circular layer of the gastric antrum. Briefly, guinea pigs were exsanguinated after being stunned. The antral part of the stomach was cut and the muscosal layer was separated from the muscle layers in $Ca^{2+}$-free physiological salt solution ($Ca^{2+}$-free

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PSS). The circular muscle layer was dissected from the longitudinal layer using fine scissors and cut into small segments (1 × 5 mm). These segments were kept in a modified Kraft-Bruhe (K-B) medium at 4 ℃ for 5 min. Then they were incubated at 36 ℃ in 4 mL digestion medium ($Ca^{2+}$-free PSS) containing 0.1 % collagenase (type I), 0.05 % dithioerythreitol, 0.05 % trypsin inhibitor, and 0.2 % bovine serum albumin for 25 – 35 min. After digestion in a shaking water bath, the softened muscle segments were transferred into the modified K-B medium, and single cells were dispersed by gentle trituration with a wide-bore fire-polished glass pipette. Isolated gastric myocytes were kept in the modified K-B medium at 4 ℃ up to 10 h.

**Electrophysiologic recording** Isolated cells were transferred to a small chamber (0.1 mL) on the stage of an inverted microscope (IX-70 Olympus, Japan) for 10 – 15 min to settle down, and then continuously superfused with isoosmotic physiological salt solution (PSS) by gravity (0.9 – 1.0 mL·min$^{-1}$). An 8-channel perfusion system (L/M-sps-8, List Electronics, Germany) was used to change the solution. Experiments were performed at room temperature (20 – 25 ℃) using the whole-cell configuration of the patch-clamp technique[5]. Patch-clamp pipettes were manufactured from borosilicate glass capillaries (GC150T-7.5, Clark Electromedical Instruments, UK) using a two-stage puller (PP-83, Narishige, Japan). The resistance of the patch pipette was 3 – 5 MΩ when filled with pipette solution. Solutions were passed through a filter of pore size of 0.2 μm before use. 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 command pulses were applied by using an IBM-compatible 486-grade computer and pCLAMP software (Version 6.02). Perforated patch recording[6] was used in some cells.

**Solutions and chemicals** Tyrode's solution contained (mmol·L$^{-1}$) NaCl 147, KCl 4, $MgCl_2 \cdot 6H_2O$ 1.05, $CaCl_2 \cdot 2H_2O$ 2, $NaH_2PO_4 \cdot 2H_2O$ 0.42, $Na_2HPO_4 \cdot 2H_2O$ 1.81, glucose 5.5 and its pH was adjusted to 7.35 with NaOH. PSS contained (mmol·L$^{-1}$) NaCl 134.8, KCl 4.5, $CaCl_2$ 2.0, glucose 5, HEPES 10 and its pH was adjusted to 7.4 with Tris [hydroxymethyl] aminomethane (TRIZMA). In $Ca^{2+}$-free PSS: $CaCl_2$ 2.0 mmol·L$^{-1}$ was omitted from PSS. Modified K-B solution contained (mmol·L$^{-1}$) *L*-glutamate 50, KCl 50, taurine 20, $KH_2PO_4$ 20, $MgCl_2$ 3, glucose 10, HEPES 10, and egtazic acid 0.5 and its pH was adjusted to 7.4 with KOH. Pipette solution contained (mmol·L$^{-1}$) potassium-aspartic acid 110, Mg-ATP 5, HEPES 5, $MgCl_2$ 1, KCl 20, egtazic acid 0.1 or 10, di-tris-creatine phosphate 2.5, disodium-creatine phosphate 2.5 and its pH was adjusted to 7.3 with KOH.

SNP (Nakarai Chemicals, Ltd, No 316-20, Tokyo, Japan), SIN-1, and spermine were dissolved prior to experiment. Methylene blue was purchased from Shenyang No 3 Chemical Reagent Plant (lot No 860501). Other reagents were purchased from Sigma (USA). TEA was made up as an aqueous stock solution (1 mol·L$^{-1}$). Nystatin was dissolved in pure $Me_2SO$ and kept at 0 ℃. All other stock solutions were kept at 4 ℃ and diluted in PSS before experiment.

**Statistical analysis** Results were expressed as $\bar{x} \pm s$, and statistical significance was tested by paired *t*-test unless stated otherwise.

## RESULTS

**Pharmacology of outward $K^+$ current of GACSMC** Membrane potential was clamped at – 60 mV using conventional whole-cell patch-clamp method, and $I_{K(Ca)}$ was elicited by step voltage command pulse from – 40 mV to + 100 mV for 400 ms with a 20-mV increment, at 10-s intervals. The $I_{K(Ca)}$ was recorded at 5 min after rupture. We monitored the capacitance current of the cell while taking samples. Once the capacitance current of a cell changed, the data of the cell were discarded. There was no significant change in the current amplitude in 40 min without treatment (data not shown). The mean amplitude of $I_{K(Ca)}$ was 1.7 nA ± 1.0 nA at 60 mV ($n = 50$). Outward currents were markedly blocked by TEA, a sensitive $I_{K(Ca)}$ blocker, under the conditions of high and low concentration of egtazic acid in pipette solutions (10 mmol·L$^{-1}$, Fig 1 A and 0.1 mmol·L$^{-1}$, Fig 1B). Charybdotoxin 200 nmol·L$^{-1}$, a specific $K_{Ca}$ channel blocker, inhibited $I_{K(Ca)}$ by 79 % ± 16 % (at 60 mV, $P < 0.01$, Fig 1C). Delayed-rectifier potassium current ($I_{KV}$) was elicited by the step voltage command pulse with $CdCl_2$ 1 mmol·L$^{-1}$ in PSS and egtazic acid 10 mmol·L$^{-1}$ in pipette solution. At 60 mV and 80 mV, the $I_{K(Ca)}$ was more markedly inhibited by TEA than $I_{KV}$ (Fig 2).

**Effect of SNP on $I_{K(Ca)}$ of GACSMC using conventional whole-cell recording** In the conventional whole-cell recording configuration, SNP 100

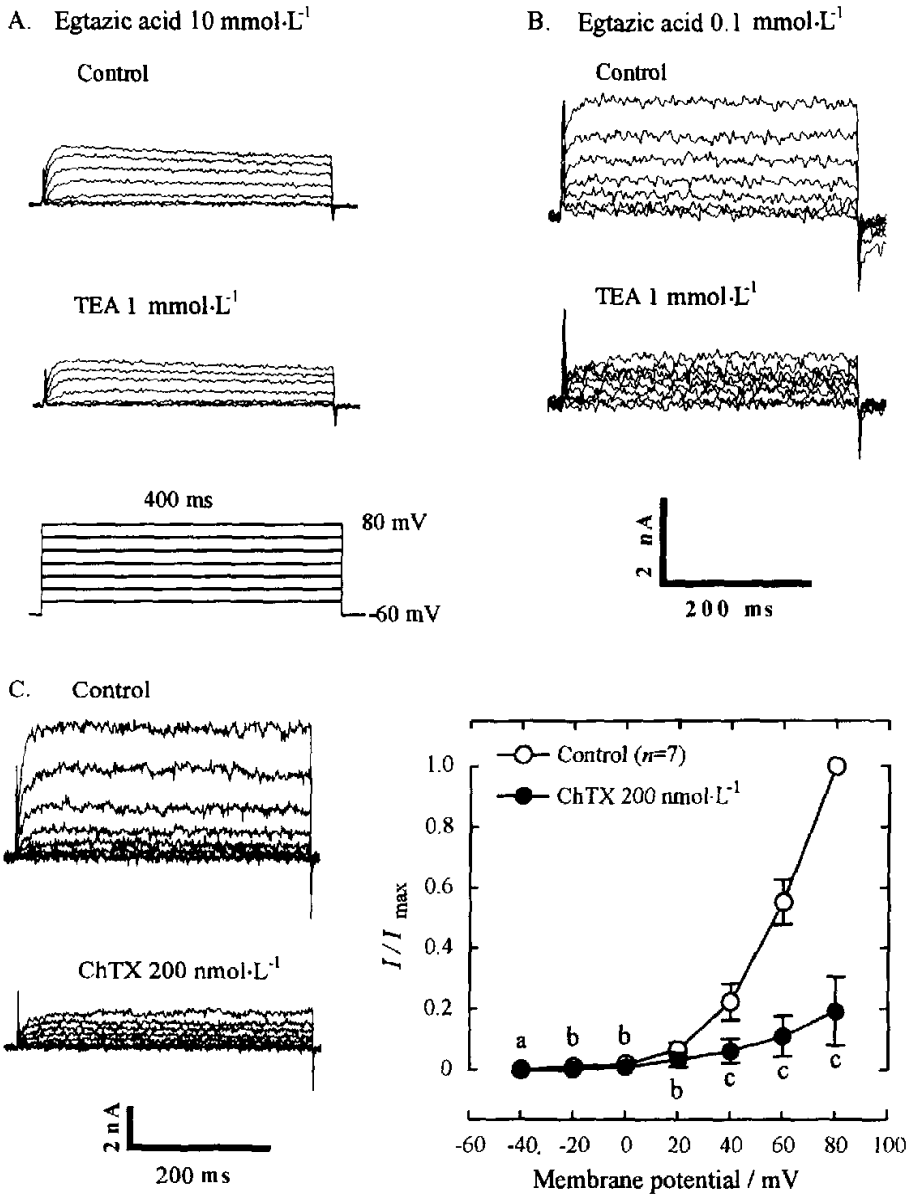


**Fig 1. Effects of TEA and ChTX on outward $K^+$ current of GACSMC in guinea pig.** $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs control*.

$\mu mol \cdot L^{-1}$ ($n = 7$) had no detectable effect on $I_{K(Ca)}$ in GACSMC at each voltage employed ($P > 0.05$, data not shown).

**Effect of SNP, SIN-1, and spermine on $I_{K(Ca)}$ of GACSMC using perforated whole cell recording** SNP (100 $\mu mol \cdot L^{-1}$) increased the amplitude of the $I_{K(Ca)}$ by 23 % ± 13 % at 60 mV (Fig 3A). As an inhibitor of soluble guanylate cyclase, methylene blue (1 $\mu mol \cdot L^{-1}$) itself did not change the amplitude of $I_{K(Ca)}$ of GACSMC in the guinea pig ($n = 13$, $P > 0.05$, data not shown). After addition of methylene blue 1 $\mu mol \cdot L^{-1}$ for 5 min, $I_{K(Ca)}$ was no longer increased by SNP 100 $\mu mol \cdot L^{-1}$ ($P > 0.05$, Fig 3B). SIN-1 200 $\mu mol \cdot L^{-1}$, another nitric oxide donor, had

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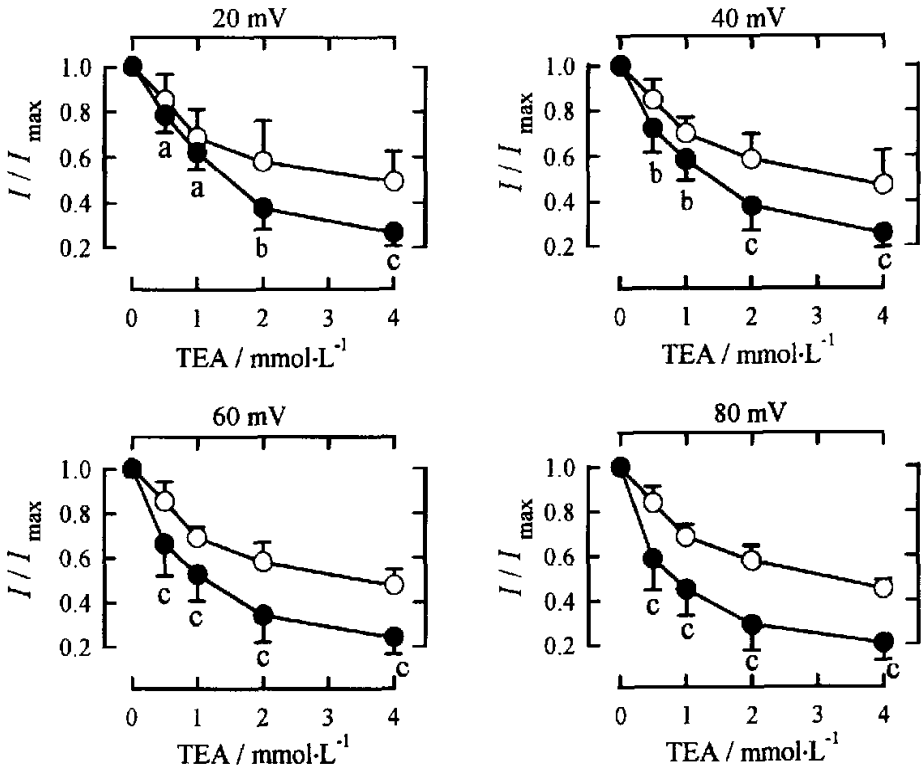


**Fig 2. Effect of TEA on $I_{KV}$(○) and $I_{K(Ca)}$(●) of GACSMC in guinea-pig. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* $I_{KV}$ using unpaired *t*-test.**

effect similar to SNP on $I_{K(Ca)}$ with an increase by 19 % ± 14 % at 60 mV (Fig 4A). SIN-1-induced $I_{K(Ca)}$ increase was also blocked by methylene blue (1 μmol·$L^{-1}$, Fig 4B). Spermine (100 μmol·$L^{-1}$) increased the magnitude of the $I_{K(Ca)}$ by 14 % ± 10 % at 60 mV (Fig 5).

## DISCUSSION

The main observation of this investigation was that three NO donors, SNP, SIN-1, and spermine, increased $I_{K(Ca)}$ in the perforated patch-clamp recording configuration but not in the conventional whole cell recording configuration. The effects of SNP and SIN-1 on $I_{K(Ca)}$ were completely blocked by methylene blue, an inhibitor of soluble guanylate cyclase.

There are 3 known types of potassium channels on smooth muscle cells[7]: calcium-activated potassium channel ($K_{Ca}$), delayed-rectifier potassium channel ($K_V$) and transient outward potassium channel ($K_{to}$). In the present study, TEA-sensitive and ChTX-sensitive large conductance $Ca^{2+}$-activated $K^+$ currents [$I_{K(Ca)}$] were a significant fraction of the outward current recorded with low egtazic acid in the pipette solution. A significant fraction of the outward current observed in the present study was TEA- and $Ca^{2+}$-sensitive. In our experimental conditions, we did not observe $I_{K(to)}$. However, Duridanova *et al*[8] found a transient outward potassium current [$I_{K(to)}$] with voltage-dependent characteristic in GACSMC of the guinea pig. There is a difference in the pharmacology between GACSMC in the guinea pig and smooth muscle cells of the rabbit basilar artery[7].

It is well known that NO inhibits motilities as nonadrenergic noncholinergic (NANC) neurotransmitters[9] in the rat stomach[1] and dog proximal colon[10]. Our previous studies have shown that exogenous NO imitate the inhibitory effects of NANC nerve on the gastric antral muscle motility in the rat[1]. Recent studies suggest that NO is released during NANC nerve stimulation, and mediates relaxation via a hyperpolarizing mechanism[9]. The channels responsible for NO-dependent membrane hyperpolarization and inhibition of gastric antral muscle

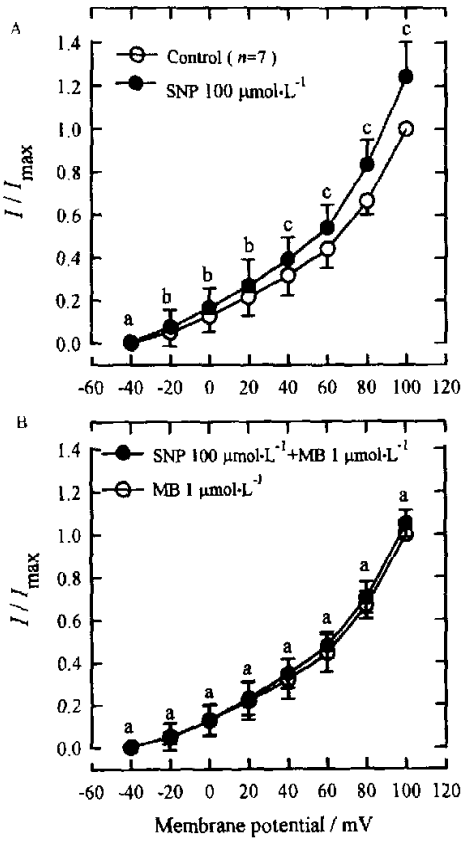


**Fig 3. Effect of SNP on $I_{K(Ca)}$ of GACSMC in guinea pig pretreated by MB. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control (A) and MB (B), respectively.**

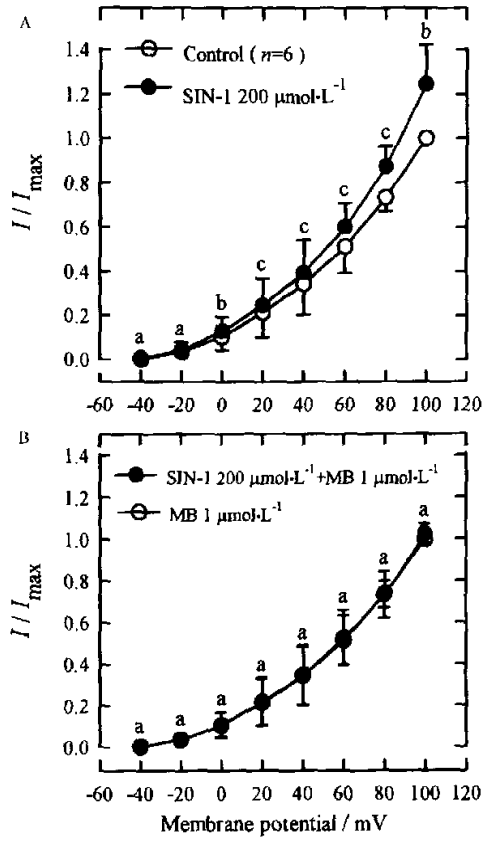


**Fig 4. Effect of SIN-1 on $I_{K(Ca)}$ of GACSMC in guinea pig in the presence of MB. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control (A) and MB (B), respectively.**

motility have not yet been identified. A number of reports show that NO-induced membrane hyperpolarization might be mediated by $K_{Ca}$ channel[2,3,11], by apamine-sensitive $K^+$ channel[12], or by two other types of $K^+$ channels ($K_{NO1}$, $K_{NO2}$)[11]. However, Zhang *et al*[4] suggest that NO-mediated hyperpolarization might be produced by suppression of calcium-dependent $Cl^-$ conductance. In the present experiment, NO donors increased $I_{K(Ca)}$ which was sensitive to TEA and charybdotoxin in GACSMC of the guinea pig using the perforated whole cell recording mode. It is most likely that NO relaxes gastric antral smooth muscle of the guinea pig through increase of $I_{K(Ca)}$.

NO relaxes smooth muscle cells by stimulation of guanylate cyclase and activation of $K^+$ channels through cyclic GMP-dependent protein kinase[2]. In single smooth muscle cells of the guinea pig stomach fundus, NO-liberating substances and cyclic GMP analogues

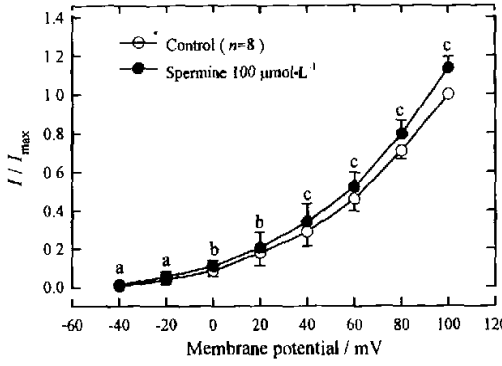


**Fig 5. Effects of spermine on $I_{K(Ca)}$ of GACSMC in guinea pig. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control.**

induced an increase in $Ca^{2+}$-sensitive $K^+$ conductivity[13]. In the present study, we have observed for the

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first time that nitric oxide increases $I_{K(Ca)}$ of gastric myocytes under perforated but not conventional whole cell recording. In the conventional whole cell recording mode, SNP did not increase $I_{K(Ca)}$ even up to 1 mmol·$L^{-1}$ (data not shown). So it did not bring a direct effect on the $K_{Ca}$ channels, as was seen in rabbit aortic cells[14]. During whole-cell recording, an important cell function disappears as a result of the loss of unknown diffusible factors into the recording pipette (washout)[15]. However, the perforated patch technique aims at retaining the cytosolic constituents. In conventional whole cell recording mode, guanylate cyclase may be diluted by pipette solution so that SNP-induced increase in $I_{K(Ca)}$ could not be seen. We found that the NO-induced increase in $I_{K(Ca)}$ was completely blocked by MB, an inhibitor of soluble guanylate cyclase. The results also suggest that SNP induced increase in $I_{K(Ca)}$ is mediated by cyclic GMP. The difference between the guinea pig gastric and the rabbit aortic artery smooth muscle might be due to species and cell type differences.

In conclusion, these data indicate that nitric oxide increase calcium-activated potassium current of gastric antral circular myocytes of the guinea pigs. The effect by NO appears to be mediated via cyclic GMP-dependent mechanisms.

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## 硝普盐、3-吗啉-悉尼酮亚胺和精胺对豚鼠胃窦环行肌钙敏感钾电流的影响[1]

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**关键词** 幽门窦；膜片箝技术；硝普盐；悉尼酮类；精胺；四乙铵；亚甲蓝

**目的**：探讨一氧化氮对豚鼠胃窦环行肌钙敏感钾电流的影响. **方法**：采用全细胞式的膜片箝方法，用精胺、硝普盐、3-吗啉-悉尼酮亚胺(SIN-1)作为一氧化氮供体. **结果**：外向钾电流被四乙铵(1 mmol·$L^{-1}$)和 ChTX (200 nmol·$L^{-1}$)显著抑制. 在穿孔膜式条件下，精胺(100 μmol·$L^{-1}$)、SIN-1 (200 μmol·$L^{-1}$)、硝普盐(100 μmol·$L^{-1}$)均增加钙敏感钾电流. 1 μmol·$L^{-1}$亚甲蓝完全阻断硝普盐与 SIN-1 导致的增加效应. **结论**：一氧化氮增加豚鼠胃窦环行肌钙敏感钾电流，该效应可能通过环磷酸鸟苷介导.

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