# Inhibitory Effect of C-Type Natriuretic Peptide on L-Type Calcium Channel Currents in Gastric Antral Myocytes of Guinea Pigs

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**Abstract.** The role of C-type natriuretic peptide (CNP) in the gastrointestinal tract is still unclear. This study was designed to investigate the effect of CNP on barium current ($I_{Ba}$) through the L-type calcium channel in gastric antral myocytes of guinea pigs. The whole-cell patch clamp technique was performed in gastric antral myocytes isolated by collagenase in guinea pigs. CNP significantly inhibited $I_{Ba}$ in a dose-dependent manner at the concentrations of 0.001, 0.01, and 0.1 $\mu$mol/l, CNP inhibited $I_{Ba}$ to 81.56 ± 2.48 %, 73.64 ± 3.65 %, and 57.77 ± 4.93 % of control at 0 mV, respectively. The values of steady-state half-inactivation voltage (33.6 ± 2.6 mV and 33.8 ± 3.4 mV, in control and CNP groups, respectively) or the half-activation voltage (−12.6 ± 2.2 mV and 12.4 ± 1.8 mV) of $I_{Ba}$ were not significantly changed ($p > 0.05$, $n = 6$). 8-br-cGMP (1 mmol/l) mimicked the effect of CNP on $I_{Ba}$, and the peak current of $I_{Ba}$ was inhibited from −403.84 ± 61.87 pA to 318.94 ± 67.17 pA ($p < 0.05$, $n = 5$). In the presence of LY83583 (0.1 $\mu$mol/l), a nonspecific inhibitor of guanylate cyclase, CNP (0.1 $\mu$mol/l)-induced inhibition of $I_{Ba}$ was partially blocked ($n = 13$, $p < 0.05$). However, when the cell was pretreated with zaprinast (0.1 $\mu$mol/l), an inhibitor of cyclic guanosine monophosphate (cGMP) sensitive phosphoesterase, the inhibitory effect of CNP on $I_{Ba}$ was significantly potentiated ($n = 11$, $p < 0.05$). KT5823 (1 $\mu$mol/l), a cGMP-dependent protein kinase (PKG) inhibitor, almost completely blocked CNP-induced inhibition of $I_{Ba}$. The results suggested that CNP can inhibit L-type calcium channel currents, and the inhibitory effect is mediated by pGC-cGMP-PKG-dependent signal pathway in gastric antral myocytes of guinea pigs.

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## Introduction

C-type natriuretic peptide (CNP), a member of the natriuretic peptides family, was first isolated in porcine brain. It is a peptide of 22 amino acid residues including 17-residue sequences flanked by two cysteine residues is common to all the natriuretic peptides (Sudoh et al. 1990). It is widely distributed and has been found in the central nervous system, cardiovascular system, digestive system, reproductive system, pulmonary system and almost all over the body (Barr et al. 1996). Natriuretic peptides elicit their physiological effects by binding to specific cell surface receptors, which have been denoted natriuretic peptide type A, B and C receptors (NPR-A, NPR-B and NPR-C). NPR-A preferentially binds atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), whereas NPR-B is more selective for CNP (Misono 2002). NPR-A and NPR-B include intracellular particulate guanylyl cyclase domains. After the natriuretic peptide binds to either receptors, intracellular cyclic guanosine monophosphate (cGMP) levels are increased. NPR-C, regarded as a clearance receptor, has a similar affinity for all three natriuretic peptides and modulates the level of them, however, this receptor subtype has no guanylyl cyclase domain (Sudoh et al. 1990; Barr et al. 1996; Anand-Srivastava 2005).

CNP has been known as a local regulator and displays a physiological and pharmacological spectrum similar to ANP and BNP (Sudoh et al. 1990; Barr et al. 1996) by binding NPR. The following functions of CNP are known at present: vasorelaxation, lowering blood pressure, anti-proliferation of vascular smooth muscle cells and cardiac fibroblasts, inhibition of hormonal secretions (luteinizing hormone, ANP and aldosterone), inhibition of basal motility of gastrointestinal tract and oviduct, and development of ovarian follicle, reductions in jejunal fluid and electrolyte secretion to keep electrolyte homeostasis (Barr et al. 1996; Anand-Srivastava 2005). Some functions are combined with the effects of CNP on ion channels. Wei et al. (1994) suggested that CNP relaxed porcine coronary arterial smooth muscle by hyperpolarization of vascular smooth muscle through potassium channel stimulation. Greger et al. (1999) showed CNP, acting *via* the second messenger cGMP, induced a marked increase in short circuit current in the rectal gland.

At present, little attention has focused on the effect of CNP on gastric smooth muscle and its ion channels mechanism. Our previous studies indicated that NPRs were distributed in the rat stomach, and CNP inhibited gastric motility in rat, guinea pigs and humans (Guo et al. 2003a,b). We also demonstrated that CNP could increase calcium-activated potassium currents in gastric antral myocytes of guinea pigs, and the process was mediated by cGMP pathway (Guo et al. 2003a). L-type calcium channel, as one of the most important ion channels in gastric myocytes, involved in regulation of gastric motility. Up to now, no reports indicated the effect of CNP on L-type calcium channel in gastric myocytes. So the aim of

present study is to investigate the effect of CNP on L-type calcium channel and its mechanism in gastric antral myocytes of guinea pig.

## Materials and Methods

*Preparation of myocytes*

Single gastric myocytes were isolated enzymatically from the antrum of guinea pigs stomach, according to a protocol derived from the method as described previously (Xu et al. 1996). Briefly, EWG/B guinea pigs (obtained from the Experimental Animal Department of Jilin University College of Medicine, Certificate No. 10–6004) of either sex weighing 300–350 g were euthanized by lethal dose of pentobarbital sodium (50 mg/kg, i.p.). The antral part of the stomach was promptly excised and equilibrated in $Ca^{2+}$-free physiological salt solution ($Ca^{2+}$-free PSS) which was oxygenated; after cutting out the mucosal layer using fine scissors, the muscle layer was separated and dissected into small segments ($1 \times 4$ mm). These segments were kept in modified Kraft–Bruhe (K-B) medium at 4 ℃ for 15 min. Then they were incubated at 36 ℃ in 4 ml digestion medium ($Ca^{2+}$-free PSS) containing 0.1 % collagenase Type II, 0.1 % dithioerythreitol (DTT), 0.15 % trypsin inhibitor and 0.2 % bovine serum albumin (BSA) for 25–35 min. After digestion, the muscle segments were transferred into the modified K-B medium, and single myocytes were dispersed by gentle agitation with a wide-bored fire-polished glass pipette. Isolated gastric antral myocytes were kept in modified K-B medium at 4 ℃ until use.

*Electrophysiological recordings*

The isolated myocytes were transferred to a small chamber on the stage of an inverted microscope IX-70 Olympus (Japan) for 10–15 min and well-attached to the bottom of the chamber, then continuously superfused with PSS (2–3 ml/min). An 8-channel perfusion system L/M-sps-8 (List Electronics, Germany) was used to change the perfusate. Experiments were performed at 20–25 ℃ and the whole-cell configuration of the patch-clamp technique was applied. The patch-clamp micropipettes were manufactured from borosilicate glass capillaries 150T-7.5 (Clark Electromedical Instruments, UK) by a two-stage puller PP-83 (Narishige, Japan). Glass pipettes (2–5 MΩ) filled with pipette solution were used to make giga seal of 3–5 GΩ. Pipette and membrane capacitance and series resistance were electronically compensated and the whole-cell currents were recorded with a patch-clamp amplifier EPC-10 (HEKA Instruments, Germany).

*Drugs and solutions*

$Ca^{2+}$-free PSS, containing (in mmol/l) NaCl 134.8, KCl 4.5, glucose 5, and N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulphonic acid) (HEPES) 10, was adjusted to pH 7.40 with tris(hydroxymethyl)aminomethane) (TRIZMA). Modified K-B medium, containing (in mmol/l) L-glutamate 50, KCl 50, taurine 20, $KH_2PO_4$ 20, $MgCl_2 \cdot 6H_2O$ 3, glucose 10, HEPES 10 and egtazic acid 0.5, was adjusted to pH

7.40 with KOH. External solution, containing (in mmol/l) NaCl 134.8, KCl 4.5, HEPES 10, $BaCl_2$ 10, $MgCl_2 \cdot 6H_2O$ 1, and glucose 10, was adjusted to pH 7.40 with Tris. Pipette solution, containing (in mmol/l) CsCl 110, tetraethylammonium 20, egtazic acid 10, HEPES 5, $Na_2ATP$ 3, and $MgCl_2 \cdot 6H_2O$ 3.5, was adjusted to pH 7.30 with Tris. CNP, LY83583, zaprinast and KT5823 were made up as stock solutions at the same concentration of 1 mmol/l. LY83583 and zaprinast were obtained from Research Biochemicals International (USA). Other reagents were purchased from Sigma Chemical Co. (USA).

*Statistical analysis*

Data were expressed as mean ± SD. Statistical significance was evaluated by Student's *t*-test and $p$ value less than 0.05 was considered significantly different.

## Results

*Effect of CNP on L-type calcium channel currents*

Barium currents ($I_{Ba}$) through the L-type calcium channel were recorded as described previously (Xu et al. 1996). Under whole-cell configuration, membrane potential was clamped at −80 mV, and $I_{Ba}$ was elicited by a single step command pulse from −80 to 0 mV for 400 ms at 10 s intervals. $I_{Ba}$ started increasing at 13.3 ± 1.3 s after cells were exposed to CNP 0.1 μmol/l (Fig. 1A). The time-course showed that $I_{Ba}$ was immediately inhibited as soon as CNP was added and within about 80 s the inhibitory effect became stabilized. The peak value of $I_{Ba}$ was decreased from −662.55 ± 4.06 pA to −395.81 ± 7.87 pA by CNP and $I_{Ba}$ recovered partially to −440.87 ± 7.81 pA after washout with normal control superfusing solution (Fig. 1A, $n = 6$). Then the membrane potential was clamped at −80 mV, and $I_{Ba}$ was elicited by step voltage command pulse from −40 to +60 mV for 440 ms with 10 mV increments at 10 s intervals. It was demonstrated that CNP significantly decreased $I_{Ba}$ at every depolarized command step potential from −20 to +40 mV in I-V relation curve (Fig. 1B, $n = 12$). CNP suppressed $I_{Ba}$ in a dose-dependant manner, and the inhibition percentages of CNP on $I_{Ba}$ at the concentrations of 0.001, 0.01 and 0.1 μmol/l were 81.56 ± 2.48 %, 73.64 ± 3.65 %, and 57.77 ± 4.93 % at 0 mV, respectively (Fig. 2, $n = 6$).

*The effect of CNP on steady-state inactivation and steady-state activation of* $I_{Ba}$

A modified double-pulse protocol was used to measure the steady-state inactivation of $I_{Ba}$ as a function of membrane potential. Prepulse potentials ranging from −100 to +40 mV were applied for a duration of 3.75 s. Following a 7 ms interpulse interval at a potential of −60 mV, the membrane potential was raised to a test potential of 0 mV for 1 s. The currents were then normalized to the current obtained at −100 mV ($I/I_{max}$) and plotted against each prepulse potential. Plotted data were well fitted by a Boltzmann equation, with a half-inactivation voltage $V_{0.5}$ of −33.6 ± 2.6 mV in control and −33.8 ± 3.4 mV in CNP group ($p > 0.05$, $n = 6$), and slope

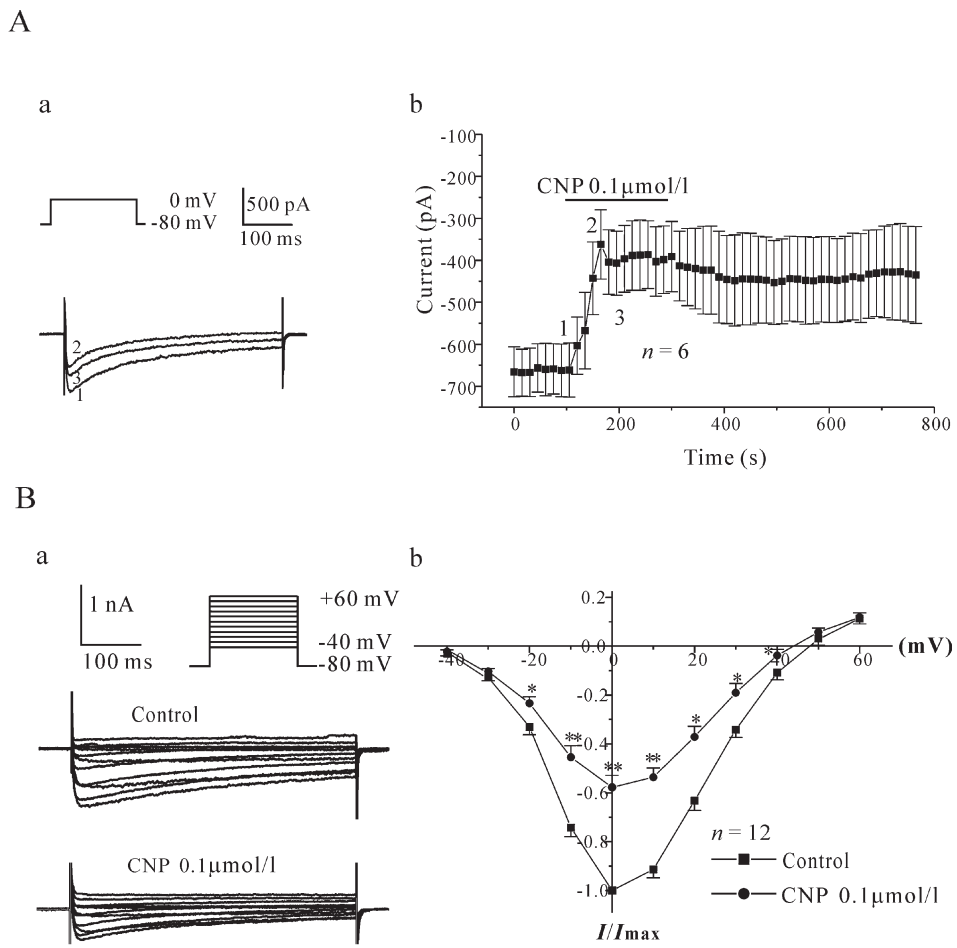



**Figure 1.** Effect of CNP on $I_{Ba}$ through the L-type calcium channel in gastric antral myocytes of guinea pigs. **A.** Membrane potential was depolarized from −80 mV to 0 mV at 10 s intervals to elicit $I_{Ba}$, while the cell was exposed to CNP (0.1 $\mu$mol/l). Aa: Raw traces of $I_{Ba}$ at 0 mV; traces 1, 2, and 3 indicate control, CNP and washout, respectively. Ab: Peak responses of $I_{Ba}$ at 0 mV by CNP ($n = 6$). **B.** Ba: Raw traces of $I_{Ba}$ elicited by step pulse. Bb: I-V relationship of $I_{Ba}$ when cell was exposed to CNP (0.1 $\mu$mol/l), $n = 12$. * $p < 0.05$, ** $p < 0.01$ *vs.* control.

factor $K$ of 9.6 ± 1.2 in control and 9.8 ± 1.4 ($p > 0.05$) in CNP group. Steady-state activation curves were estimated from the peak conductance at each potential by using the following equation: $I_{Ba} = g_{Ba} \cdot (V - V_{rev})$ where $g_{Ba}$, $V$ and $V_{rev}$ are peak conductance, test potential and observed reversal potential, respectively. The value of half-activation was −12.6 ± 2.8 mV in control and −12.4 ± 1.8 mV in

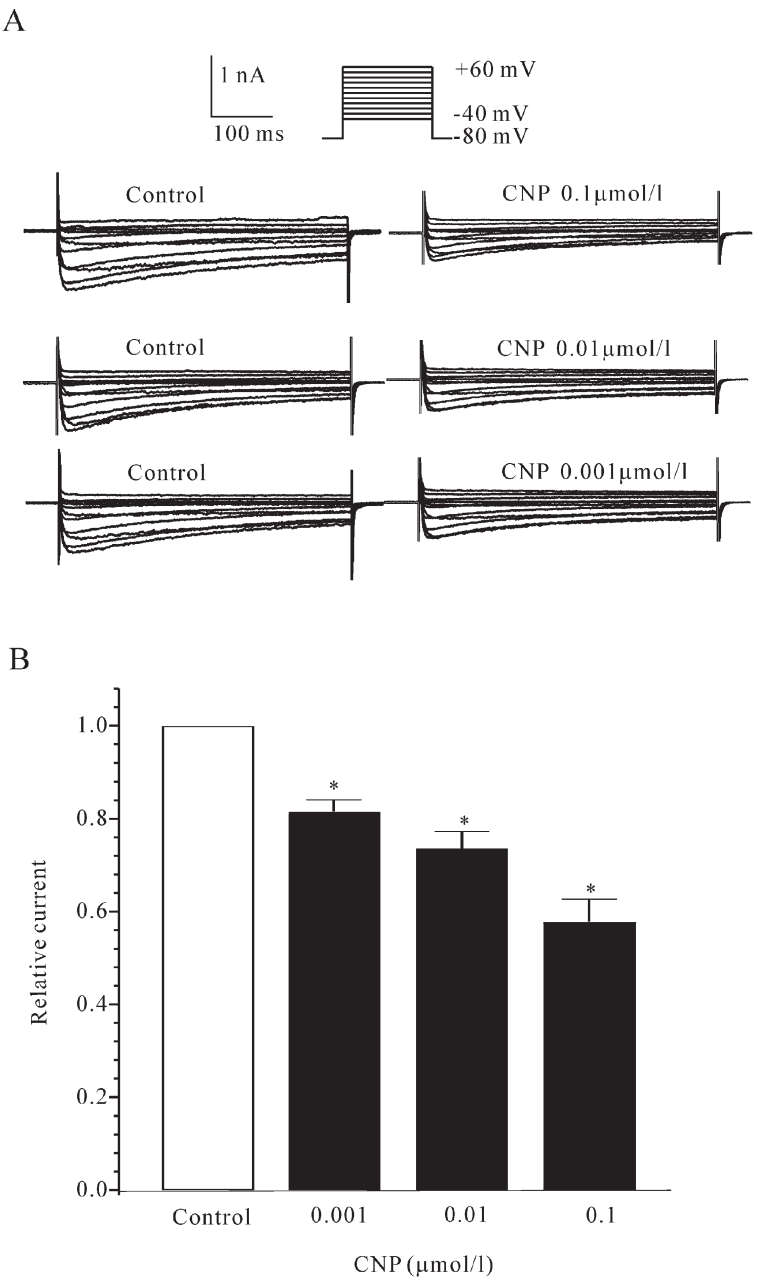



**Figure 2.** Dose-dependant relationship between the concentrations of CNP and $I_{Ba}$ in gastric antral myocytes of guinea pigs. **A.** Raw traces of $I_{Ba}$ elicited by step pulse. **B.** Peak values of $I_{Ba}$ at 0 mV normalized and averaged for cells exposed to CNP (0.1, 0.01, 0.001 $\mu$mol/l, respectively). * $p < 0.01$ *vs.* control.

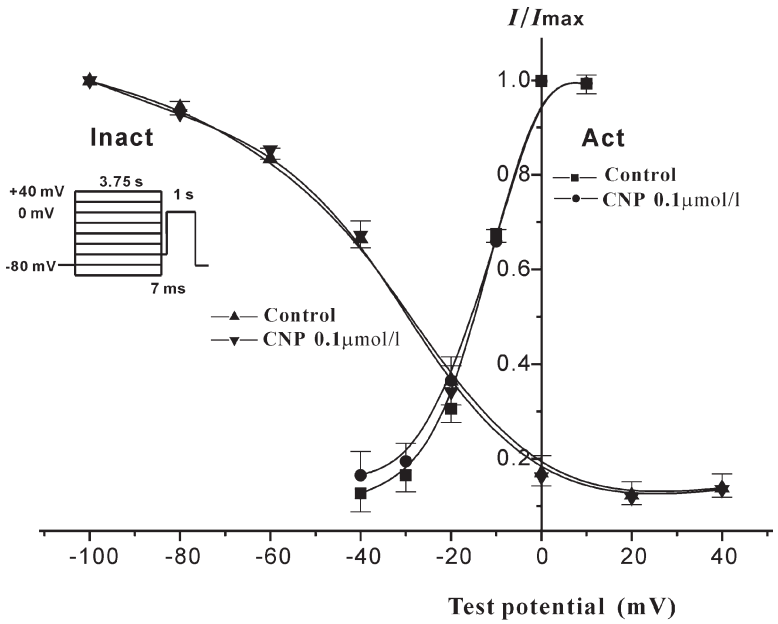



**Figure 3.** Steady-state activation (Act) and inactivation (Inact) curves for the cells exposed to CNP. For the steady-state activation relationship, peak conductance was determined from the peak inward currents, corrected for the change in driving force at each of the test potentials and normalized to 1. Driving force was obtained from the difference between the test potential and the observed reversal potential. For the steady-state inactivation relationship, peak currents were obtained using a two-pulse protocol (3.75 s of prepulse potential from −100 to +40 mV) followed by a 7 ms interpulse interval at −60 mV, the membrane potential was rised to a test potential of 0 mV for 1 s. The difference between peak current and late current present before the end of the test pulse was normalized to 1 and plotted against the prepulse potential.

CNP group ($p > 0.05$) with slope factors $K$ of 6.8 ± 0.3 and 6.5 ± 0.2 ($p > 0.05$) in control and CNP groups (Fig. 3).

*The relationship between effect of CNP on $I_{Ba}$ and cGMP*

Since NPR-B is more selective for CNP and NPR-B includes intracellular particulate guanylyl cyclase domains, it was observed whether cGMP mimicked the effect of CNP on L-type calcium currents. 8-br-cGMP, a cell membrane permeable cGMP, significantly suppressed $I_{Ba}$ (Fig. 4A,B), 1 mmol/l 8-br-cGMP inhibited the peak current of $I_{Ba}$ from −403.84 ± 61.87 pA to −318.94 ± 67.17 pA (Fig. 4C, $p < 0.05$, $n = 5$).

To determine whether the inhibition of $I_{Ba}$ induced by CNP was mediated by NPR-B coupled with guanylate cyclase, LY83583, an inhibitor of guanylate cyclase was used. In the I-V relation curve it was demonstrated that CNP significantly

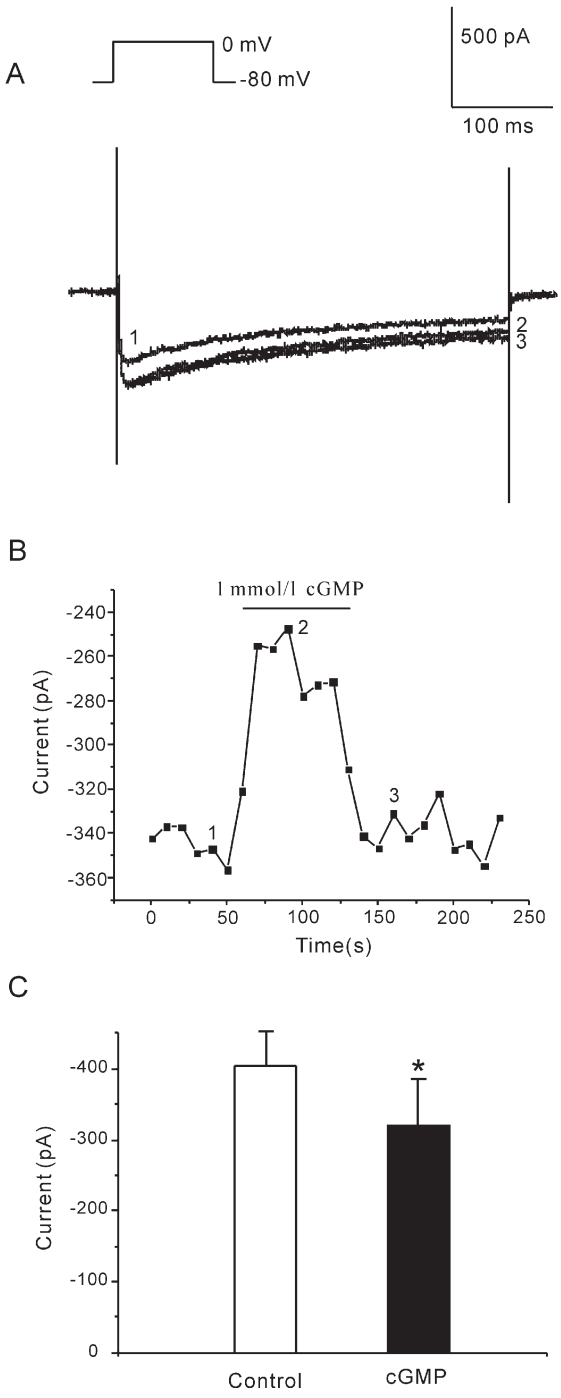
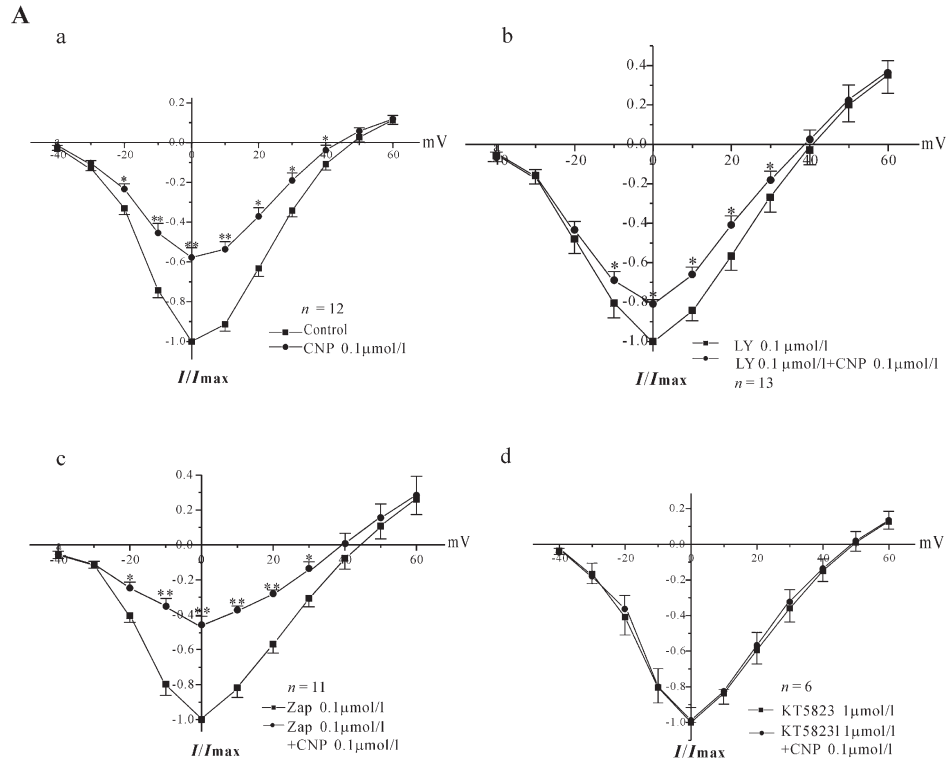



**Figure 4.** Effect of 8-Br-cGMP on $I_{Ba}$. Membrane potential was depolarized from −80 to 0 mV at 10 s intervals to elicit $I_{Ba}$, while the cell was exposed to 8-Br-cGMP (1 mmol/l). **A.** Raw traces of $I_{Ba}$ at 0 mV; traces 1, 2, and 3 indicate control, 8-Br-cGMP and washout, respectively. **B.** Peak responses of $I_{Ba}$ at 0 mV by 8-Br-cGMP. **C.** Summary of $I_{Ba}$ elicited by single step pulse from −80 mV to 0 mV. $n = 5$, * $p < 0.05$, *vs.* control.



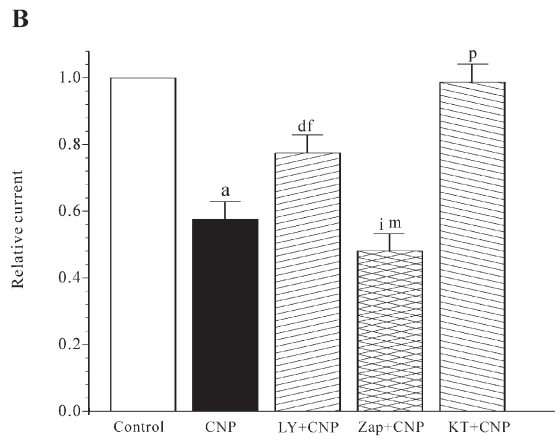




**Figure 5.** Relationship between cGMP and CNP-induced inhibition of $I_{Ba}$. **A.** I-V relationships of $I_{Ba}$. Aa: I-V curve for control and cells exposed to CNP ($\mu$mol/l). Ab: I-V curve for control and cells exposed to CNP ($\mu$mol/l) in the presence of LY83583 (LY), an inhibitor of guanylate cyclase ($n = 10$, * $p < 0.05$, ** $p < 0.01$ *vs.* control). Ac: I-V curve for control and cells exposed to CNP ($\mu$mol/l) in the presence of zaparinast (Zap) ($n = 8$, * $p < 0.05$, ** $p < 0.01$ *vs.* control). Ad: I-V curves for control and cells exposed to CNP ($\mu$mol/l) in the presence of KT5823, a cGMP-PKG inhibitor ($n = 6$, $p > 0.05$ *vs.* control). **B.** Peak values of $I_{Ba}$ at 0 mV normalized and averaged for cell exposed to CNP and under the conditions of pretreatment with LY, Zap and KT5823, respectively. [a] $p < 0.01$ *vs.* control; [d] $p < 0.01$ *vs.* LY group; [f] $p < 0.01$ *vs.* CNP group; [i] $p < 0.01$ *vs.* Zap group; [m] $p < 0.01$ *vs.* CNP group; [p] $p < 0.01$ *vs.* CNP group.

suppressed $I_{Ba}$ at every depolarized command step potential from −20 to +40 mV before pretreatment with LY83583 (Fig. 5Aa, $n = 13$). After pretreatment with LY83583 (0.1 $\mu$mol/l) for 8–10 min, the inhibitory effect of CNP (0.1 $\mu$mol/l) on $I_{Ba}$ was significantly weakened (Fig. 5Ab), and the relative current was increased from 0.59 ± 0.05 to 0.81 ± 0.055 at 0 mV ($p < 0.01$, $n = 10$, Fig. 5B). To further investigate effect of intracellular cGMP level on CNP-induced inhibition, zaprinast, an inhibitor of cGMP sensitive phosphoesterase was used. In the I-V relation curve it was demonstrated that zaprinast (0.1 $\mu$mol/l) significantly potentiated CNP-induced inhibition of $I_{Ba}$ (Fig. 5Ac), and the relative current was decreased from 0.59 ± 0.05 to 0.47 ± 0.052 at 0 mV ($p < 0.01$, $n = 11$, Fig. 5B). To investigate the effect of cGMP generated by CNP on $I_{Ba}$, KT5823, a cGMP-dependent protein kinase (PKG) inhibitor, was employed. After pretreatment with KT5823 (1 $\mu$mol/l), the inhibition of $I_{Ba}$ induced by CNP was almost completely blocked, and there was no significant difference between the KT5823 and KT5823 plus CNP groups (Fig. 5Ad and 5B, $n = 6$, $p > 0.05$ *vs.* control group).

## Discussion

Although the concentration of CNP in peripheral tissue was much lower than in the brain, CNP was found in rat stomach, ileum-jejunum and colon-caecum by radioimmunoassay, and CNP mRNA was detected in ileum-jejunum by Northern blot analysis (Minamino et al. 1993). Kim et al. (2004) observed the immunoreactive CNP in rabbit colon extracts by a specific radioimmunoassy, the presence of CNP mRNA in the colonic tissue by RT-PCR, and immunoreactive CNP was two-fold higher in the mucosal layer of colon than in the muscular layer. The effects of CNP on gastrointestinal motility have only been described by some reports; relaxant effect on chick rectum muscle strip, inhibitory effect on rat tenia coli, and relaxation effect on guinea pig caecum circular smooth muscle (Itaba et al. 2004). Our previous study demonstrated that CNP relaxed gastric smooth muscles in guinea pig, rat and human stomach (Guo et al. 2003a,b), and indicated CNP activated calcium-activated potassium currents in guinea pig gastric smooth muscle cells.

Since L-type calcium channels play an important role in gastrointestinal smooth muscle contraction, the effect of CNP on L-type calcium current was investigated in the present study. CNP significantly inhibited L-type calcium currents in a dose-dependent manner in gastric myocytes of guinea pig. However, CNP did not change the steady-state inactivation and steady-state activation curves. The results suggest CNP-induced inhibition of L-type calcium currents to be one of mechanisms, by which CNP relaxes gastric smooth muscle. Similar effects of CNP on calcium channel currents were also observed in many different tissues, for example, CNP has a potent and selective inhibitory effect on L-type calcium channel in magnocellular neurosecretory cells (Rose et al. 2005), mouse sinoatrial node (Rose et al. 2004), bullfrog atrial myocytes (Rose et al. 2005). Under physiological conditions, the inhibition of T-type calcium channels may be involved in the inhibition of the aldosterone secretion induced by ANP (Barrett et al. 1991). All of the above

studies suggest that CNP plays a down regulatory role in L-type calcium channel currents of all excitory cells.

CNP exerts its biological functions by interacting with the NPRs. NPR-A and NPR-B can increase the production of intracellular cGMP. It was reported that CNP caused dose-dependent increases in cGMP production in the gastric and colonic tissues (Kim et al. 2004), and our previous study (Guo et al. 2003b) illustrated that cGMP was markedly increased by CNP in rat gastric smooth muscle tissue and perfusion solution. cGMP, as one of the intracellular second messengers, plays an important role in regulating ion channels. L-type $Ca^{2+}$ channel currents were inhibited by 8-Br-cGMP in cultured rat mesenteric artery smooth muscle cells (Taguchi et al. 1997) and guinea pig ventricular myocytes (Ziolo et al. 2003), and the activation of the ANP receptor reduced calcium current *via* guanylate cyclase-dependent cGMP production in human atrial cells (Le Grand et al. 1992). It was demonstrated that ANP inhibits the cardiac L-type calcium channel activity through the intracellular production of cGMP and then activation of protein kinase (Tohse et al. 1995). Additionally, by activating PKG to phosphorylate proteins, it also affects the ion channels. Wang et al. (2000) suggested that stimulatory effects of cGMP on $I_{Ca}$ in rabbit atrial cells were likely to be mediated *via* PKG-dependent phosphorylation of calcium channels or associated proteins.

In our present study, 8-Br-cGMP, a cell membrane permeable cGMP, can mimic the effect of CNP on $I_{Ba}$, and LY83583, an inhibitor of guanylate cyclase, attenuated CNP-induced inhibition of $I_{Ba}$ while zaparinast, an inhibitor of cGMP sensitive phosphoesterase, potentiated CNP-induced inhibition of $I_{Ba}$. From above results, it was concluded that the NPR-B and cGMP were involved in CNP-induced inhibition of $I_{Ba}$. To determine whether the activity of PKG is responsible for the CNP-induced inhibition of $I_{Ba}$, KT5823, an inhibitor of PKG, was tested. After pretreatment with KT5823 for 8–10 min, the CNP-induced inhibition of $I_{Ba}$ was almost completely blocked. CNP firstly binds with NPR-B and activates membrane-bound guanylate cyclase (mGC), or particulate guanylate cyclase (pGC) and stimulates cGMP production. However, LY83583 is a non-selective inhibitor or more specific for soluble guanylate cyclase (von Bulow et al. 2005; Yamada et al. 2006) than mGC, so CNP-induced inhibition of $I_{Ba}$ was only partially blocked by LY83583. Our results suggest that the effect of CNP on $I_{Ba}$ is mediated by pGC-cGMP-PKG pathway in gastric smooth muscle of guinea pig.

NPR-C is regarded mainly as a clearance receptor to modulate the level of natriuretic peptides in the circulation without activating the intracellular second messenger cascades (Barr et al. 1996). But, some researchers have indicated that NPR-C is coupled to adenylyl cyclase inhibition or phospholipase C activation through inhibitory guanine nucleotide regulatory protein and also mediates additional actions of the natriuretic peptides (Anand-Srivastava et al. 2005). Rose et al. (2003, 2004, 2005) showed CNP to be able to strongly inhibit L-type calcium currents in bullfrog atrial myocytes, rat magnocellular neurosecretory cells and mouse sinoatrial node and these effects were mediated by NPR-C. In our present study, the effect of CNP on L-type calcium channel current exhibited that peak

current was rapidly inhibited and the inhibitory effect was sustained for long time, however, the peak current was rapidly inhibited and recovered in the presence of 8-Br-cGMP. The results suggest further studies to determine whether the rapid inhibitory effect of CNP on L-type calcium channel current may be related to cGMP generation by NPR-B while the sustained inhibition may be mediated by NPR-C.

In summary, CNP inhibited L-type calcium channel currents *via* pGC-cGMP-PKG pathway and it is one of the mechanisms by which CNP plays an inhibitory regulating role in gastric smooth muscle of guinea pig.

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